THE ROLE OF THE FIBROBLAST IN THE HUMAN PREGNANT CERVIX

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ABSTRACT

There are extensive alterations to cervical structure and function throughout pregnancy and labour. The cervical deep stromal layer is the target for many of these structural alterations. The underlying mechanisms of cervical change in pregnancy are unresolved, yet clinical interventions are in common usage. There are limitations with vaginal administration of current cervical ripening agents with varied absorption rates and unpredictable effects. A small study of potential direct clinical benefit is performed to assess the capability of a needle-free injection device to administer a solution to the human uterine cervix. The principle of cervical needleless injection is proven with a mean depth of penetration to 5.6mm. There could be major benefits in dose accuracy and speed of effect due to the direct nature of administration, but whether this is sufficient to be clinically advantageous is questionable.

Key component of the cervical stroma, the fibroblast, possesses many of the properties required to facilitate the cervical changes seen throughout pregnancy. Little is known about the phenotypic alteration and functions of the commonest resident cervical cell, the fibroblast, during pregnancy and labour. Decidualisation of endometrial stromal cells has been extensively investigated occurs under the influence of progesterone, with the added requirement of cAMP levels in-vitro. The cervix, like the endometrium is exposed to this progesterone-rich environs during pregnancy, and like the endometrium undergoes significant structural re-arrangement during this period. The hypothesis that a decidual-like phenotypic variation in cervical stromal cells exists is tested. Cervical ripening in the last few weeks of pregnancy has also been likened to an inflammatory reaction with invasion of leukocytes, activation of proinflammatory cytokines and of connective tissue degrading matrix metalloproteinases. Prior studies have observed upregulation of collagen and proteoglycan degrading MMPs in the labouring cervix. However if leukocyte extravasation is a key component to cervical remodelling at term, then MMP-2, whose main substrate is collagen type IV i.e. basement membrane, would provide a mechanism to facilitate this. ProMMP-2 requires activation by MMP-14,

known to be regulated by prostaglandin in the human monocyte, but previously unrecognised in the human cervix.

Human cervical biopsies were divided as untreated samples and homogenised for RNA extraction, or prepared as cultured human cervical stromal cells (CSC) and treated with medroxyprogesterone acetate and cAMP elevating agents for 6 and 10 days. Messenger RNA expression by quantitative RT-PCR and/ or protein release by ELISA was performed for decidualisation markers PRL, IGFBP-1, TF, VEGF, desmin, laminin and fibronectin, and receptor expression PR, EP2 and EP4. IL-8 gene expression and protein release was compared under decidual stimulus. MMP-14 mRNA expression in cervical explants, cultured stromal cells, and a monocyte population was compared under prostaglandin E_2 and MCP-1 stimulus. Cervical explants from the first trimester demonstrate the major decidualisation markers with x14-fold prolactin and x58-fold IGFBP-1 mRNA expression, relative to non-pregnant explants. CSCs under the influence of progesterone and cyclic-AMP, from both pregnant and non-pregnant women, display a "decidual-like" morphological alteration, and markedly increased mRNA expression (pregnant: PRL 26 ± 5 s.e.m, IGFBP-1 559 ± 282 s.e.m, $p< 0.01$) and protein release (PRL x 6-fold, IGFBP-1 x 25-fold, $p< 0.01$) relative to control. CSCs exposed to decidual stimuli show reduced IL-8 mRNA expression (0.39 ± 0.04, $p< 0.01$) and protein release ($p< 0.01$). MMP-14 is expressed by the human cervix and CSC but is not upregulated by prostaglandin E_2 as demonstrated in a monocyte population (x 5-fold ± 0.6).

This altered cervical fibroblast phenotype has extensive implications in the study of the mechanism of pregnancy associated cervical remodelling, possibly serving as protective mechanism of cervical integrity until functional progesterone withdrawal.

DECLARATION

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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ABBREVIATIONS

AA	arachidonic acid
ACTH	adrenocorticotrophin
AF	amniotic fluid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BrcAMP	8-bromo-cyclic adenosine-3',5'-monophophate
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
cDNA	complementary DNA
cGMP	cyclic guanine monophopshate
CD	cellular differentiation marker
COX-1/2	cyclo-oxygenase-1/2(synonym PGHS)
CRH	corticotrophin releasing hormone
CSC	cervical stromal cell
CSM	cell separation medium
Ct	threshold cycle
DAR	donkey anti-rabbit
DEPC	diethyl pyrocarbonate
dH_20	distilled water
DHEAS	dehydroepiandrosterone sulphate
DM	decidualising mix (MPA, 8-bromo-cAMP, E2)
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DSC	decidual stromal cell
E2	oestradiol
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EP2	prostaglandin E_2 type 2 receptor

EP4	prostaglandin E_2 type 4 receptor
ER	oestrogen receptor
FACS	fluorescence activated cell sorter
FAM	6-carboxy-fluorescein
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
GDP	guanine diphosphate
GPCR	G-protein coupled receptor
GTP	guanine triphosphate
GM-CSF	granulocyte macrophage colony stimulating factor
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HPA	hypothalamo-pituitary-adrenal axis
IC	intracellular
ICAM	inflammatory cell adhesion molecule
IFN	interferon
IGFBP-1	insulin-like growth factor-binding protein-1
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
IOL	induction of labour
IP_3	inositol 1,4,5-triphosphate
IUD	intrauterine death
KDR	type 2 receptor of VEGF
LAVH	laparoscopic assisted vaginal hysterectomy
LHRH	luteinising hormone releasing hormone
LMP	last menstrual period
LPS	lipopolysaccharide
LUS	lower uterine segment
MCP-1	monocyte chemotactic protein-1
MLCK	myosin light chain kinase

MMP	matrix metalloproteinase
MoxB	methyl oximating B solution
MPA	6α-methyl-17α-hydroxyprogesterone acetate
mRNA	messenger RNA
mw	molecular weight
NADPH	nicotinamide adenine diphosphate
NAF	neutrophil activating factor
NBF	neutral buffered formalin
NFκB	nuclear factor kappa B
NK	natural killer
NP	non-pregnant
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSB	non-specific binding
P4	progesterone
PAF	platelet activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDEIV	phosphodiesterase inhibitor type 4
PG	prostaglandin
PGE	prostaglandin E
PGDH	prostaglandin dehydrogenase
PGES	prostaglandin E synthase
PGFS	prostaglandin F synthase
PGHS	prostaglandin H endoperoxidase synthase (see COX)
PGI_2	prostacyclin
PKA	protein kinase A
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
PR-A/B	progesterone receptor-A/B
PRL	prolactin

PROM	preterm premature rupture of membranes
PRM	Prostaglandin E_2, Rolipram, MPA regime
PTL	preterm labour
Q-RT-PCR	quantitative-reverse transcription-PCR
RNA	ribonucleic acid
RT	reverse transcription
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute Medium
RU486	mifepristone
s.e.m	standard error of the mean
TAH	total abdominal hysterectomy
TBS	tris buffered saline
TIMP	tissue inhibitor of metalloproteinases
TF	tissue factor
7-TMB	seven transmembrane
TNFα	tumour necrosis factor-α
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor

CHAPTER 1

LITERATURE REVIEW

1.1 PARTURITION

1.1.1 The Physiology of Parturition

The physiological process of parturition is complex and, although most cases proceed without complication, we do not yet fully understand the underlying biological mechanisms. Our ability to intervene effectively where necessary is thus limited. The process of human labour has been well characterised in terms of regular uterine contractions resulting in effacement and dilatation of the cervix allowing passage of the mature fetus to the outside world. Prior to this the myometrium gradually becomes more responsive to stimuli over months, the cervix "ripens" over several weeks and eventually, effectors initiate the process of labour (see Fig 1.1). It is this synchronous preparatory process and its regulators that must be unravelled.

The complexities of this process are numerous. The fetal organs must mature appropriately for independent existence, the uterus and cervix must have undergone the preparatory phases pre-labour for optimal response to uterine contractility stimuli, and the maternal lactation requirements must have been triggered to sustain the newborn. All these integrated systems must be orchestrated with precision for a successful outcome. This review will focus on the human cervix and the changes seen in pregnancy and labour.

The preparatory process named cervical ripening has been described in humans as taking place over several weeks in late gestation. In reality it is perhaps a continuum of changes that are seen in early and throughout pregnancy. During this time the cervix undergoes tissue remodelling resulting a softer, more compliant organ that will efface and dilate in response to myometrial contractions and mechanical stretch by the presenting part of the fetus. The structure of the non-pregnant cervix will be discussed in detail together with the changes then seen before and during labour. Mediators influencing this process are varied but prostaglandins are currently the favoured therapeutic agent exploited in clinical practice. Nitric oxide donors, relaxin, antiprogestogens, oestrogen, and cytokines (IL-8 & IL-1) have been

successful cervical ripening agents in human, rabbit, rat and guinea-pig studies and the search for the ideal therapeutic tool continues (Radestad *et al* 1990; Calder *et al* 1993; Chwalisz *et al* 1994; El Maradny *et al* 1994; Chwalisz *et al* 1997; Thomson *et al* 1997b; Elliott *et al* 1998; Luque *et al* 1998; Shi *et al* 2000).

The endocrine system in pregnancy has been investigated for decades but systemic and local changes have now been distinguished. In general it appeared that increased systemic prostaglandins, progesterone (P4) withdrawal, and the increased oestrogen: progesterone ratio (E2:P4 ratio) are responsible for maintenance or progression of labour rather than the initiation. These factors are known to enhance myometrial activity by promoting gap junction formation, oxytocin receptors and increasing the agonist response.

Undoubtedly prostaglandins are important in parturition, and increased peripartum production in fetal membranes, with locally attenuated catabolism, have supported this across several species (Keirse 1979; Mitchell 1987b; Geirsson *et al* 1990; Lundin-Schiller *et al* 1990; Kelly *et al* 1992; Van Meir *et al* 1997). Prostaglandins are produced in many tissues including fetal membranes, myometrium, cervix and macrophages. Cytokines, particularly interleukin-8 (IL-8) are also produced in abundance by these cells. Prostaglandins are certainly involved in myometrial responsiveness and cervical ripening but appear to represent a final common pathway in parturition rather than an early event in initiation. Control of prostaglandin levels in parturition may be controlled in part by the catabolic enzyme prostaglandin 15-dehydrogenase (PGDH) since levels of this enzyme are lower in the membranes overlying the cervix (Van Meir *et al* 1997). These variations seen in PGDH activity/ expression are particular to the chorion but are not reflected in placentae. Moreover, infection of the membranes leads to a decrease in the levels of PGDH and thus will be permissive of prostaglandin actions (van Meir *et al* 1997).

Fig 1.1 *Timetable of events in preparation for labour*

	MAJOR CHANGES			SUCCESSFUL LABOUR
UTERUS	↑Coupling ↑Ion channels ↑Receptors ↓NO system	→	↑Conductivity ↑Excitability ↓Relaxation	**Reinforcement of contractions**
	37 weeks to term			
CERVIX	↑Inflammatory response ↑Collagenolysis	→	↑Ripening	**Dilatation**
	25 weeks to term			
FM	↑ECM degradation	→	↓Tissue integrity	**Rupture**
	25 weeks to term			
STEPS	**CONDITIONING**			*ACTIVE LABOUR*

(adapted from Garfield et al 1998)

Immune cells, macrophages and neutrophils in particular, are prevalent in the uterus throughout the menstrual cycle, implantation, pregnancy and more so at parturition (De *et al* 1991; Hunt 1994; Hunt *et al* 2000). The possible roles for these cells will be explored.

Recent studies have explored a neuro-immune hypothesis based on the increased innervation seen in the cervix at term concurrent with macrophage migration. It is possible that local neuropeptide release has a chemoattractant role but has not yet been investigated. Rat studies have shown than cervical denervation by transection disrupts parturition (Renegar *et al* 1992). Without doubt cervical remodelling takes place well in advance of any changes in uterine contractility and cervical changes in labour are associated with immune cell recruitment, activation and enzymatic degradation. The interaction between immune mediators (PGs, iNOS and cytokines) is complex and must integrate endocrine, paracrine and possibly neural pathways.

The average human pregnancy lasts 40 weeks from the first day of the last menstrual period, yet due to advances in perinatal care infants can survive from 24 weeks gestation whether delivered spontaneously or by intervention. Despite this, preterm delivery accounts for at least 75% of perinatal mortality (Slattery *et al* 2002). Pregnancy can also be prolonged for several weeks although recommendations suggest delivery by 42 weeks (RCOG Clinical effectiveness support group 2001). There is therefore a wide variation in gestation at delivery in humans. This we combat largely by relying on advancing neonatal care, but have little means of intervention to predict or prevent preterm delivery or to initiate labour artificially. Many avenues explored attempt to intervene at the end of a gradual and prolonged cascade initiated several days or weeks beforehand. For example, tocolytics of all kinds may be successful in delaying delivery for a matter of hours or days but none have improved perinatal outcomes (Gyetvai *et al* 1999; Worldwide Atosiban versus Beta-agonists Study Group 2001). Is this "last gasp rescue" just too late to reverse all the interactive mechanisms that have gone before, and perhaps by this stage it is not in the interests of that fetus to remain in an environment that could be increasingly dangerous. Similarly, is it any surprise that when pregnancy is

prolonged and our efforts focus on emulating the final events when the preparatory phases are not complete, that we sometimes fail and produce dysfunctional labour?

1.1.2 Evidence from Animal Models

Several species-specific theories exist regarding the initiation of labour including mechanical distension of the uterus, progesterone withdrawal in sheep and rodents, oxytocin stimulation, maturation of the fetal HPA axis and decidual activation. In fact it is probably a result of all of these elements combined. There does however appear to be distinct mechanisms in term and preterm labour where, in the latter, the fetoplacental unit fires the trigger early to escape an increasingly hostile environment. In cases such as infection or placental insufficiency or abruption, the immense stimulation of cytokines and prostaglandins (PG) supercedes the physiological control mechanisms of pregnancy maintenance. In idiopathic preterm labour, there is no such sign of fetal compromise and other explanation must be sought.

Primates do share a similar reproductive tract and a similar placental structure but some species, such as the baboon, differ in corticotrophin releasing hormone (CRH) and oestrogen secretion throughout pregnancy (Smith *et al* 2002). Studies in baboons have suggested a marked regional up-regulation in COX-2 mRNA expression in lower uterine, cervical and decidual tissues compared to the fundus, placenta and amnion (Wu *et al* 2000). This suggests a site-specific activation of prostaglandin synthesis with a temporal relationship to increased myometrial contractility. Gorillas and chimpanzees most closely mirror human events but studies with these animals are difficult for ethical reasons and in no species does implantation occur to the depth seen in humans.

In sheep, interruption of the HPA-axis results in the failure of labour. Labour is driven by fetal HPA-axis maturation, hypothalamic corticotrophin releasing hormone (CRH) and ACTH release causing raised fetal adrenal cortisol levels which in turn

cause an increased oestrogen synthesis by the placenta, via increased 17α-hydroxylase-17,20-lyase activity, with a concurrent reduction in progesterone production. This results in both prostaglandin synthesis and induction of contraction-associated proteins (CAPs), and so labour ensues (Lundin-Schiller *et al* 1990). More recently the control of peripartum prostaglandin production has been brought into question as this seems to occur before the rise in fetal and maternal oestrogen, but concurrent with the fetal plasma cortisol levels, and placental prostaglandin synthase/ cyclooxygenase-2 (COX-2) expression. The hypothesis that there are two separate pathways of intrauterine PG production is supported by the work of Whittle et al (Whittle *et al* 2000). This proposes a cortisol-dependent fetal placental trophoblast pathway directly increasing fetal plasma PGE_2, and an E2-dependent pathway in maternal intrauterine non-trophoblast tissues directly up-regulating $PGF_{2\alpha}$ production and stimulating uterine activity. Progesterone withdrawal may be a prerequisite but although in humans this is not a systemic effect, there may be local variation in progesterone activity in intrauterine and cervical tissues. Ovine PGDH activity increases in labour (Riley *et al* 2000) but the reverse has been shown in humans and the control of PGDH itself is multifactorial (Challis *et al* 1999).

Luteolysis induced progesterone withdrawal occurs in other animals (e.g. mouse, rat) but again this event is absent in humans where progesterone production shifts from the corpus luteum to placenta. Indeed prostaglandin F receptor (FP) or COX-2 deficient mice have shown reproductive failure in both early pregnancy and parturition (Lim *et al* 1997; Sugimoto *et al* 1997) and this could be as a result of the lack of prostaglandin induced P4 withdrawal. Interestingly, 5α-reductase is an enzyme that inactivates progesterone and Mahendroo et al reported the important discovery that the 5α-reductase knockout mouse will labour but does not undergo cervical ripening (Mahendroo *et al* 1999). Delivery can then expedited by ovariectomy which imitates a luteolytic progesterone fall, or by administration of relaxin. This shows that there are differing mechanisms controlling the various components of parturition. Rat studies have confirmed the interaction of

progesterone withdrawal, cytokines, leukocyte recruitment, NO and prostaglandins in cervical ripening (Shi *et al* 2000).

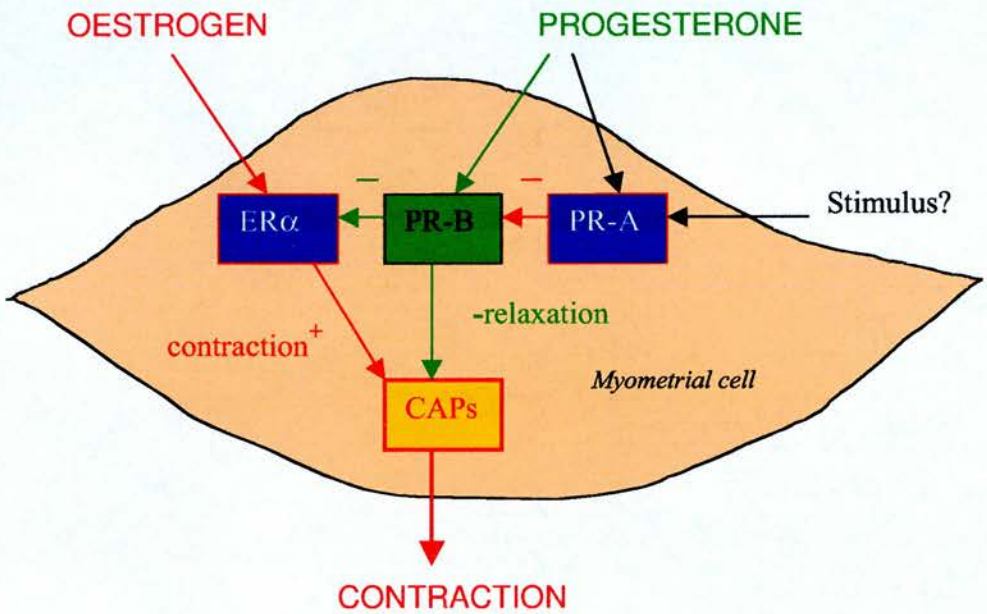
Guinea pigs have similarities to the endocrinology of humans as progesterone secretion shifts from corpus luteum to placenta and remains high throughout gestation (Elger *et al* 1987). Chwalisz et al have demonstrated the key role of antiprogestins in cervical softening in guinea pigs and have had reasonable success initiating cervical ripening with cytokines and NO (Chwalisz *et al* 1994; Chwalisz *et al* 1997).

1.1.3 Human Initiation of Labour

The necessity for an intact HPA axis appears not to be essential for humans as anencephalic fetuses can be spontaneously delivered around term, as can fetuses with adrenal hypoplasia. Endogenous placental cortisol production is impossible as the necessary hydroxylases are absent in humans. Nor in humans is there the prelabour circulatory progesterone withdrawal, although there may be subtle local intrauterine changes in progesterone receptor status.

In human labour, progesterone receptors (PR-A/ PR-B ratio) and oestrogen receptor isoform (ERα) increase in myometrium and lower uterine segment reducing functional progesterone responsiveness (Smith *et al* 2002; Winkler *et al* 2002). PR-A in human myometrial cells is a repressor of PR-B acitivity (Pieber *et al* 2001). This also increases oestrogen activation and correspondingly COX-2 and oxytocin receptor (OTr) mRNA encouraging a contractile state. See Figure 1.2.

Figure 1.2 *Myometrial progesterone/ oestrogen receptor interaction*

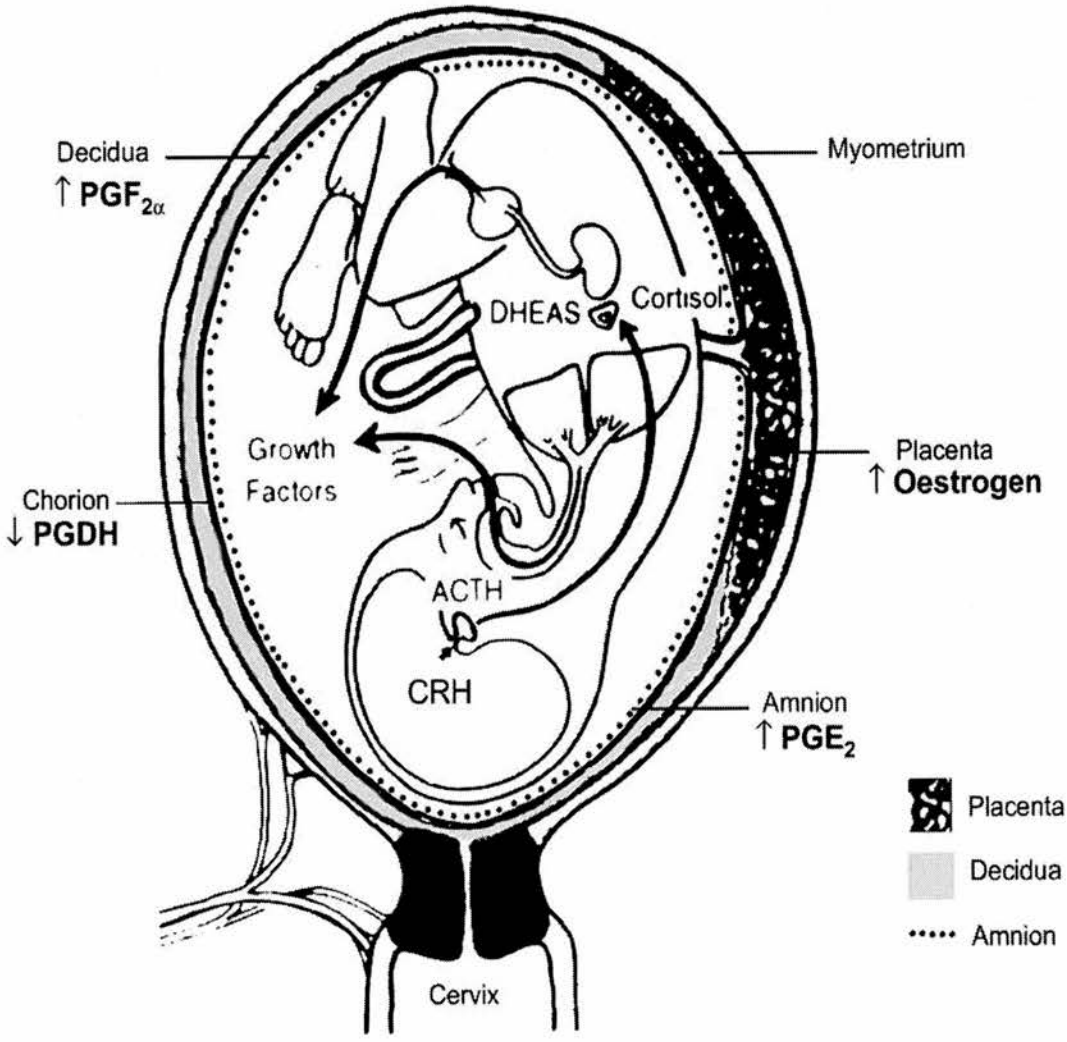


Adapted from (Smith *et al* 2002)

Maternal plasma cortisol rises in late pregnancy, CRH increases exponentially throughout gestation and is related to timing of delivery with a high second trimester value strongly associated with preterm labour (McLean *et al* 1999). Prelabour, CRH-binding protein falls thereby increasing free CRH concentration. CRH receptors are present in maternal pituitary and myometrium, and fetal pituitary and adrenal, thereby providing a mechanism to influence fetal adrenal androgen synthesis and oestrogen concentrations. The myometrial CRH receptor expressed throughout pregnancy is different to that seen at term and this may reflect a change in function from relaxation to contraction (Lopez Bernal *et al* 1995). The differing roles of hypothalamic and placental CRH in parturition are still under investigation.

In the human fetus the effect of the cortisol surge may have a different emphasis, to promote key enzymes responsible for fetal lung maturation and preparing other organs to meet the demands of extra-uterine function (see Fig 1.3). Recent publications would support the postulation of fetal HPA maturation being the primary trigger. Whittle et al in their review of the glucocorticoid regulation of parturition discuss the central role of cortisol, both its direct prostaglandin effects and its indirect intrauterine CRH mediated effects, stimulating COX-2 expression and initiating human labour (Whittle *et al* 2001). Cortisol from fetal membranes may also serve to alter PGDH activity by competitively displacing progesterone from GR with down-regulated PGDH transcription (Challis *et al* 1999). The human mechanism is different to that of sheep in that it is adrenal androgen production that drives the HPA axis as opposed to a placental source. Dehydroepiandrosterone sulphate (DHEAS) is then converted by placental sulphatase and aromatase to oestradiol. So, a similar mechanism may operate in sheep and humans after all, with the exception of the androgen precursor source, where humans rely on fetal and maternal adrenal supplies to provide the term rise in intrauterine oestrogen, without a drop in progesterone.

Figure 1.3 *Initiation of labour*



1.2 CLINICAL ASPECTS OF ABNORMAL PARTURITION

Failure of the timely process of parturition results in prolonged pregnancy with its increased risk of perinatal mortality. Premature initiation results in preterm labour. Greater understanding of parturition would lead to safe, effective interventions for both prolonged pregnancy and preterm labour.

1.2.1 Preterm Delivery

Preterm delivery is defined as delivery between 20 and 37 completed weeks gestation. Prematurity is associated with 60-80% of all perinatal deaths excluding congenital anomalies, the disproportionate majority occurring in the small group delivered before 32 weeks gestation (Goldenberg 2002; Slattery *et al* 2002). This reflects the increased severity of complications of prematurity at earlier gestations. Between 24 and 28 weeks gestation, perinatal survival increases from 40 to 80% in developed countries with neonatal facilities, whereas survival approaches 100% at 32 weeks gestation (Slattery *et al* 2002). Perinatal morbidity has long term sequelae for those affected and is positively associated with both preterm delivery and low birth weight. At extreme prematurity (24 weeks), only 12 % of those babies born alive will survive without disability by the age of 30 months, corrected to expected date of delivery (Wood *et al* 2000). And 23-24% of those delivered before 25 weeks gestation that survive to discharge, will have severe disability (Tin *et al* 1997; Wood *et al* 2000). Cerebral palsy, neurological and developmental disability and chronic lung disease are a few of the many health problems affecting these individuals and their families. Not only are families affected by physical disability and dependence, but these children often display behavioural dysfunction and poor school performance, which may limit their options in adult life. Accompanying these direct effects, there are also wider economic considerations for health services (Kilpatrick *et al* 1997) and society as a whole.

Although, with good neonatal facilities, survival is much improved, the prevalence of preterm delivery is increasing with variable reported rates from 6-15% (Slattery *et al* 2002) depending on the population studied. The current UK rate of preterm labour is 7-9% (Gardosi *et al* 2000; Aveyard *et al* 2002). The higher risk group, delivered before 32 weeks gestation, accounts for 1-2 % of all deliveries. Approximately half of these are due to preterm labour, another 25% result from preterm premature rupture of the membranes (PROM) and the rest are attributable to obstetric intervention (Tucker *et al* 1991). The observed rise is difficult to explain and varies in different ethnic groups, but the increasing use of assisted conception and resultant multiple pregnancies cannot be ignored. Multiple pregnancies have a substantial risk (40-50%) of preterm delivery, but a proportion of this is due to increased intervention beyond 34 weeks in an attempt to reduce late losses. The higher the order of pregnancy, the shorter the average gestation at delivery; 36 weeks gestation is average for twins compared with 33 weeks gestation for triplets.

Idiopathic preterm labour is multifactorial with risk factors such as social disadvantage, multiple pregnancy, ethnic origin, substance misuse, age, parity and previous reproductive history interacting. The pathogenesis of preterm labour is not well understood, and it is often not clear whether preterm labour represents early idiopathic activation of the normal labour process or results from a pathologic mechanism.

The pathway to the initiation of preterm labour probably involves premature decidual activation. Although decidual activation may be mediated in part by the fetal-decidual paracrine system, in many cases of preterm labour, it appears that this activation occurs in association with occult upper genital tract infection. Infection has been widely investigated as a cause of preterm labour, and while it is known that any maternal systemic infection may initiate labour, it is less clear whether intra-uterine or genital tract infection has a significant role. There is a current hypothesis that sub-clinical choriodecidual inflammation may be responsible (Sebire 2001).

1.2.2 Induction of Labour

Delivery beyond 42 weeks gestation occurs in 3.5-12% of pregnancies (Lagrew *et al* 1986; Sue-A-Quan *et al* 1999; Rand *et al* 2000). Prolonged gestation, defined as > 294 days from last menstrual period, is associated with increased perinatal mortality, where late intra-uterine death is a particularly tragic outcome for an otherwise uncomplicated pregnancy. Although the gestation specific risk of perinatal death with prematurity is high, when considered relative to total ongoing pregnancies, the risk is highest at term and post-dates. The rate of stillbirth or perinatal death rises seven-fold, from 0.7 per 1000 ongoing pregnancies at 37 weeks gestation to 4.8 per 1000 at 42 weeks gestation (Hilder *et al* 1998).

Perinatal mortality statistics show that the risk of unexplained intra-uterine death increases significantly beyond 41 weeks (Yudkin *et al* 1987; Hilder *et al* 1998; Cotzias *et al* 1999). The identification of the fetuses at risk has eluded us, despite various methods of antenatal monitoring, and so routine induction of labour serves as intervention in an attempt to reduce these late deaths. There has been hesitation to introduce routine elective induction prior to 42 weeks due to the reported increased rates of intervention and caesarean delivery without improvement in perinatal outcome (Prysak *et al* 1998; Seyb *et al* 1999; Alexander *et al* 2000; Maslow *et al* 2000). This has now been questioned as evidence grows that elective induction after 41weeks does not increase caesarean section rates (Hannah *et al* 1992; Parry *et al* 1998; Sue-A-Quan *et al* 1999; Rand *et al* 2000; RCOG Clinical effectiveness support group 2001). Several studies have also shown that accurate dating by ultrasound (Gardosi *et al* 1997), and clear departmental guidelines (Harris *et al* 2000) can reduce the number of pregnancies requiring induction for postmaturity.

Induction of labour is a common intervention in post-dates and complicated pregnancies. It occurs in approximately 20% of all pregnancies in the UK and in excess of this in some regions (Gardosi *et al* 1997; Robson *et al* 1997; Nuutila *et al* 1999; MacDorman *et al* 2002). The commonest indication for induction of labour is post-dates pregnancy, in 60-70% of cases. However routine induction in nulliparous

women at term has been associated with increased caesarean section rates (Prysak *et al* 1998; Seyb *et al* 1999; Maslow *et al* 2000). It must therefore be recognised that there is still a balance of risks to achieve, so each case should be judged individually and induction should not be considered entirely risk free.

Although much progress has been made in the last 40 years and current methods are reasonably successful, difficulties are still encountered. These range from lack of efficacy, in a small sub-group of women, to unpleasant side effects and dangerous complications such as uterine rupture. Uterine rupture can have catastrophic results and is a particular risk for those women who have previously had a caesarean section where the risk of perinatal death is estimated 0.02-0.05% (Plaut *et al* 1999; Zelop *et al* 1999; Lydon-Rochelle *et al* 2001; Smith *et al* 2004).

Induction of labour is defined as any intervention designed to artificially initiate cervical ripening and/or uterine contractions leading to progressive dilatation and effacement of the cervix with the purpose of vaginal delivery. Favoured methods of induction of labour at present are artificial rupture of membranes, vaginal prostaglandins and intravenous syntocinon infusion. Each of these methods has its limitations and complications so the search is still on to find the ideal agent.

It is now a well-established fact that unripe cervices lead to prolonged labour with associated complications of maternal pyrexia, sepsis and postpartum haemorrhage, failed induction, an increased risk of caesarean delivery and fetal effects such as poor Apgar scores, birth asphyxia and neonatal sepsis (Calder 1979; Edwards *et al* 2000). PGE_2 produces cervical ripening as well as relaxing the cervical smooth muscle and contracting the myometrium. Dinoprostone (PGE_2) is currently the agent of choice, either as a vaginal/intracervical gel or a slow release polymer insert, but a requirement for special storage and the relatively high cost of these preparations can be prohibitive for some countries.

Misoprostol, a PGE_1 analogue is an inexpensive alternative and simplifies storage requirements, but there are licensing and dosage issues, despite many studies

supporting its efficacy and safety. Developed for the prevention of NSAID induced peptic ulcers, misoprostol was designed to resist catabolism by 15-OH PGDH and was always known to have abortifacient properties. After development for use in termination, the first assessment of misoprostol as a labour induction agent were in 1992. Misoprostol has been repeatedly shown to effectively induce labour, resulting in improved cervical score, shortened delivery interval and reduced requirement for other oxytocics (Danielian *et al* 1999; Hofmeyr *et al* 1999; Wing 1999; Sanchez-Ramos *et al* 2000). Yet the ideal regime has not been established due to concerns about its uterotonic effect and association with hyperstimulation and higher rates of meconium stained liquor (Goldberg *et al* 2001). Vaginally administered misoprostol has different pharmacokinetics to oral, with a slower and lower peak plasma concentration which may be sustained for up to four hours (Sanchez-Ramos *et al* 2000; Hofmeyr *et al* 2001). This results in a greater exposure overall while avoiding the surge seen with oral administration. The result is greater efficacy when considering delivery outcomes against time, (How *et al* 2001; Kwon *et al* 2001) but concern remains with fetal safety. Currently lower dose regimes are being assessed. Misoprostol has repeatedly been shown to be as efficacious if not more, than dinoprostone but sufficient safety data has yet to be established, particularly in those women with a uterine scar (Kolderup *et al* 1999; Rowlands *et al* 2001; Rozenberg *et al* 2001). Clearly, until the safety issues are clarified this therapy will not be universally welcomed.

The antigestagen mifepristone (see p32) at a dose of 200mg given 24, 48 or 72 hours before induction of labour results in a slightly shorter delivery interval and a reduced requirement for either prostaglandins or oxytocin, but not the more dramatic effect we see in early pregnancy (Elliott *et al* 1998; Wing *et al* 2000).

Uterine rupture is a rare but catastrophic complication of labour following a previous caesarean delivery, and can result in fetal death with serious maternal morbidity. Induction of labour is always performed with caution in these cases, but restricted prostaglandin use has been successful. The incidence of uterine rupture is 0.2-1.5% depending on the definition used (Sanchez- Ramos *et al* 2000). There is an

associated uterine dehiscence where scar separation is noted incidentally at caesarean section or post delivery where there have been no clinical signs or complications. An American retrospective cohort analysis reports the overall uterine rupture rate at 0.45% in women with one previous caesarean delivery and found an increased rate with labour, labour induction and labour induction with PGE_2. The risk with repeat caesarean prelabour was 0.16% increasing to 0.52%, 0.77% and 2.45% respectively (Lydon-Rochelle *et al* 2001). This reflects findings in other studies and reinforces the concern that any agent that also stimulates myometrial activity is not ideal for cervical ripening (Zelop *et al* 1999; Smith *et al* 2004). Smith's population based study confirms the increased risk of uterine rupture with perinatal death where the woman has had no previous vaginal birth and is induced with prostaglandins (Smith *et al* 2004).

Uterine disruption has been reported in 5.6% women with previous caesarean deliveries, treated with misoprostol compared to only 0.2% in those not exposed (Plaut *et al* 1999). However uterine rupture has also been reported in parous women without prior uterine surgery (Bennett 1997). Currently there is not enough data to support safety of misoprostol use in those women with a scarred uterus until enough properly conducted controlled studies have been completed.

1.2.3 Clinical Markers of Cervical Ripening

Many authors have commented on the importance of cervical assessment before induction of labour and several methods, from examination to biochemical markers or ultrasound assessment, have been explored. The most cost effective and widely used system is that of the Bishop score (Edwards *et al* 2000), or a modified version (Calder *et al* 1974), where five cervical parameters are scored and the total reflects ripeness. A low score is unfavourable and equips the clinician with pertinent information allowing decisions regarding suitability and method of induction.

Fetal fibronectin is an extracellular matrix protein produced by choriodecidual cells and can be found in cervical secretions in advance of labour. Indeed fetal fibronectin correlates well to cervical ripening at term (Ekman *et al* 1995) and is detectable in cervical secretions of those at risk of preterm labour. It is thought to reflect choriodecidual separation at internal os, but does not add to the clinical findings at term. A randomized controlled clinical trial did not show any reduction in preterm labour for asymptomatic fibronectin positive women treated with antibiotics (Andrews *et al* 2003). Although fibronectin has not proved to be of any benefit in predicting or preventing preterm labour, it can provide reassurance for some due to its high negative predictive value (Hellemans *et al* 1995).

Transvaginal sonography (TVS) can detect several signs of potential cervical incompetence or preterm labour: Firstly, dilatation of the internal os - this can be seen prior to dilatation of the external os detectable on digitial vaginal examination. Secondly, funnelling - prolapse of membranes into the cervical canal with shortened closed cervical length. Again this is visible prior to external os dilatation, either spontaneously or with fundal pressure. Thirdly, shortening of the cervix - the length of the endocervical canal seen in apposition.

TVS of cervical length has low positive predictive value for low risk women (Iams *et al* 1996), but may be a useful adjunct in managing high risk cases to select those requiring cervical cerclage. Retrospective studies have confirmed an association

with reduced endocervical canal length and prior preterm labour (Guzman *et al* 1998a), and a longitudinal study showed that this can be detected between 16 and 24 weeks' gestation (Guzman *et al* 1998b). Cervical length has been shown to be the best indicator of subsequent preterm delivery (Rozenberg *et al* 2002) with 48% of women with a cervical length ≤15mm at 22 weeks gestation delivering before 32 weeks (Hassan *et al* 2000). The negative predictive value of 96.7% is also reassuring in this group, but limitations for use as a screening tool in low risk women arise with a low sensitivity of 8.2%. Even though high risk cases can be identified, selection for cervical cerclage based on shortened cervical length < 15mm has not been shown to reduce subsequent preterm delivery in a randomized controlled trial (To *et al* 2004). Outwith screening where there is symptomatic evidence of threatened preterm labour, cervical length < 15mm is the best predictor of subsequent preterm delivery within 7 days ; 37% vs 0.7% with Cx length > 15mm (Tsoi *et al* 2003).

Outwith clinical practice, other methods have been used to assess cervical state. Fluorescence spectroscopy can inform molecular and physical structure and has been used to examine collagen content in various tissues. Garfield et al have used this technology in the form of a collascope to assess cervical tissue changes in pregnancy in both rats and humans, the advantage being the ability to perform longitudinal studies in the same subjects. Light-induced autofluorescence (LIF) in the cervix is thought to originate from pyridinoline, a major crosslink in collagen fibril structure, and was seen in rats to reduce in late gestation and after treatment with antiprogesterone (Garfield *et al* 2001). These findings were corroborated by parallel cervical resistance studies and electron microscopy and the changes spontaneously reversed. Human studies have shown a similar decline in the last 15 weeks of pregnancy with slow postpartum recovery, and may prove clinically useful in prediction of preterm labour (Maul *et al* 2003). The non-invasive nature of LIF may aid future investigations in cervical function in addition to potential benefits in labour management.

1.2.4 Termination of Pregnancy

Around 192,000 terminations of pregnancy procedures are performed in the UK each year at present (RCOG Guideline Development Group 2001). There are two main methods of termination of pregnancy, surgical and medical. Surgical, largely vacuum aspiration and medical, induced abortion techniques have both benefited from the use of prostaglandins. Indeed prostaglandin use has so dramatically changed medical methods to the extent that where it was once a rare alternative to vacuum aspiration, it is now commonplace and in many cases preferred.

It is known that myometrial stimulation occurs in all trimesters with exogenous prostaglandin administration (Mitchell 1987b). It has also been shown that prostaglandins are synthesized in the cervix itself and prostaglandin receptor (mostly EP2) presence in the cervix has been demonstrated (Uldbjerg *et al* 1987; Smith *et al* 1998). Therefore, termination of pregnancy either medically or surgically in the first trimester, and medically at any gestation can be facilitated by prostaglandin use.

Those analogues in use initially had success rates of 94-100% (Norman 1992). The 1980's saw virtual universal use of vaginal prostaglandin analogues, mostly gemeprost, both for medical abortion and cervical ripening pre-surgical vacuum aspiration. More recently, the discovery of mifepristone and misoprostol has further improved the efficacy of medically induced abortion.

Mifepristone is an antiprogestin that competes with progesterone at the receptor level thus producing a reduced progesterone effect, and sensitising the uterus to prostaglandins (Kelly *et al* 1990). Csapo's theory that progesterone withdrawal results in uterine activity is borne out here, despite many studies being unable to show a physiological reduction in progesterone in normal term pregnancy (Csapo 1956; Csapo *et al* 1965). Not only is uterine activity stimulated after approximately 36hours, but increased myometrial sensitivity to prostaglandins also results. The exact mechanism of action is not known but increased leukocyte and monocytes numbers, with elastase and MMP stimulation is seen (Denison *et al* 2000).

Mifepristone alone is not a particularly effective abortifacient (success rates of 60-70%) (Cameron *et al* 1986; Bygdeman 1993), but in combination with prostaglandin analogues results in successful termination of pregnancy in 92-99% of cases (Silvestre *et al* 1990; Norman *et al* 1991; McKinley *et al* 1993; Peyron 1993; Baird *et al* 1995; Ashok *et al* 1998b; Spitz *et al* 1998; Schaff *et al* 1999; Schaff *et al* 2000). Mifepristone is also helpful in reducing side effects as the prostaglandin dose required can be significantly reduced.

Misoprostol has been exploited for the precise purposes of therapeutic termination of pregnancy with great success (Norman *et al* 1991; Peyron 1993; Jain *et al* 1994; el-Refaey *et al* 1995). Although there has been recent controversy surrounding its use in pregnancy in terms of licensing (Friedman 2001; Gebhardt 2001; Hale *et al* 2001; Mackenzie 2001; Wagner 2001), misoprostol has been extensively evaluated (Goldberg *et al* 2001) and introduced into routine clinical practice for termination of pregnancy.

Mifepristone alone is relatively free of side effects, but did not approach the efficacy of prostaglandins (Cameron *et al* 1986). A combined approach allowed a reduction in prostaglandin dosage and attendant side effects, with equivalent efficacy of 94-100% (Cameron *et al* 1986; Rodger *et al* 1989; Silvestre *et al* 1990; UK Multicentre Trial 1990).

Misoprostol has been explored as a prostaglandin alternative since 1991 and has been shown to be extremely effective (McKinley *et al* 1993; Peyron 1993; Spitz *et al* 1998) with the advantage or oral administration. Paradoxically many investigators are now using misoprostol vaginally as efficacy improves in the 7-9 week group (Ashok *et al* 1998b; Schaff *et al* 1999; Schaff *et al* 2000). Complete abortion rates of 97.5% have been achieved in women up to 63 days pregnant, and 95% at 9-13 weeks have been achieved using a combination of mifepristone and vaginal misoprostol, with 93% of women treated as day cases (Ashok *et al* 1998a; Ashok *et al* 1998b).

Similarly, mifepristone and misoprostol use has now extended to managing silent or incomplete miscarriage, giving women an option to avoid surgical intervention or

prolonged conservative management. Equally in second trimester terminations, abortion within 24hrs can be achieved in 90-97% of cases depending on the misoprostol regime (Goldberg *et al* 2001).

Surgical vacuum aspirations are performed throughout the first trimester. Cervical priming before surgical termination of pregnancy reduces the operative morbidity associated with aspiration procedures, by reducing the force required to achieve adequate cervical dilatation and thereby minimising the incidence of uterine perforation, cervical damage, false passages and incomplete evacuation. The same problem lies with the usual side effects seen with prostaglandin administration, those of abdominal pain, nausea, vomiting and diarrhoea (Ledingham *et al* 2001a). Vaginal misoprostol has been proven at least as effective with the added benefits of a reduced side effect profile and much reduced expense (el-Refaey *et al* 1994). Clinical trials have found 400μg misoprostol vaginally three hours prior to surgery to be the optimal regime (Fong *et al* 1998; Henry *et al* 1999).

Following success in guinea-pigs (Chwalisz *et al* 1997), the nitric oxide donors have been evaluated as a cervical ripening agent in first trimester termination of pregnancy in humans (Thomson *et al* 1997b; Thomson *et al* 1998; Facchinetti *et al* 2000). The advantage of nitric oxide would be that it is known to be a smooth muscle relaxant and so should not cause the same the side effects as prostaglandins and may therefore be safer. Results showed less effective ripening with increased intra-operative blood loss, but a much improved side effect profile than PGE_2 (Thomson *et al* 1998). However the blood loss was no greater than a control group of parous women. Use of sodium nitroprusside may be more effective than isosorbide mononitrate and appears to reduce the rate of headache (Facchinetti *et al* 2000). This may with further assessment be a useful adjunct to current methods, but the myometrial effect requires more attention, particularly if this is be extrapolated to term pregnancy and the frequency and acceptability of headache must be established.

An ideal induction agent would cause cervical ripening without uterine activity, allowing this process to complete uninterrupted prior to the initiation of active

labour, and without any unwanted side effects. As there are so many mechanisms involved and their mediators often have wide-ranging effects, the difficulty is with the specificity of effect. Many of the previously mentioned mediators such as cytokines and nitric oxide are under investigation as the search continues. It may well be that a combined approach may be the way forward in optimising our clinical procedure. Clearly prostaglandins are a major factor in parturition and they have already contributed greatly to our understanding of this process and provided useful clinical tools. The challenge is to further define the role of specific prostaglandins and their receptors, in combination with the many inflammatory mediators known to be involved.

1.3 THE HUMAN UTERINE CERVIX

1.3.1 Cervical Changes in Pregnancy

Before labour begins, the human uterine cervix undergoes some structural changes. Both physiologically and therapeutically, this is referred to as cervical ripening and applies to the process whereby the cervix undergoes significant connective tissue remodelling resulting in a compliant and distensible organ. Cervical remodelling takes place gradually throughout pregnancy with "cervical ripening" occurring in the latter stage, before the dramatic cervical changes seen during labour. Ledger et al confirmed that this cervical change was an active process when softening was shown in cervices, surgically separated from the uterus, where the effects could not be a passive result of uterine contractions (Ledger *et al* 1985).

Although comparisons have been made between many aspects of parturition and the inflammatory process, it is perhaps cervical ripening that provides the most compelling evidence. PGE and NO are both vasoactive and may also have effects in allowing leukocyte ingress. Activated macrophages and circulating leukocytes release pro-inflammatory chemotactic cytokines, up-regulating adhesion molecules and leukocyte recruitment. Leukocyte degranulation releases MMPs, degrades collagen, activates fibroblasts to release MMPs specific to basement membrane, further enhancing leukocyte migration, degranulation and enzymatic degradation of collagen. The result is remodelled tissue that is more compliant and which readily dilates in response to mechanical stretch and uterine activity. The evidence for this hypothesis will be examined.

1.3.2 The Non-pregnant Cervical Structure

To understand the changes occurring in pregnancy and labour one must first appreciate the cervical structure in the non-pregnant state. The nature of cervical tissue was well described by Danforth in 1947 (Danforth 1947). The cervix is bounded proximally by the uterine body, at the internal os, and distally, by the external os and vagina. Distinct from the muscular uterine corpus, the cervix is a fibrous organ with less than 15% of its content contributed by smooth muscle, mostly found peripherally and increasingly abundant nearer to the corpus (Danforth 1954). The remaining extracellular matrix consists of dense collagen (66% type I, 33% type III) fibril bundles and small numbers of elastin fibres, with intervening ground substance (Kleissl *et al* 1978). Type IV collagen was only found in the basement membrane region in non-pregnant cervices (Minamoto *et al* 1987). The endocervical canal is lined by tall columnar epithelium, with highly branched glands, down to the external os where the squamo-columnar junction marks the transition to stratified squamous epithelium, which is continuous with that of the vagina.

Danforth reported that in the non-pregnant state, elastin constitutes less than 1 percent of cervical tissue, largely located perivascularly and sparsely scattered. Subsequent technologies have allowed for this to be further refined and suggest that these elastin fibres run between the epithelium and smooth muscle cells in a longitudinal direction, from external to internal os. At this proximal point there is a slightly higher ratio of elastin to collagen, relative to the more distal portion (Leppert 1995). In the uterine body, elastin remains a small component, sparsely distributed, although largely in the outer third.

Smooth muscle cells account for 10-15% of cervical tissue, largely placed peripherally and maximally near the internal os. Near the histological internal os is the fibromuscular junction, above which smooth muscle becomes the dominant component of the uterine body, and this transition is variable from gradual to abrupt (Danforth 1947).

The fibroblast or stromal cell is the main cellular constituent in the human cervix as described by Junqueira (Junqueira *et al* 1980) and supported by evidence in other species (Hegele-Hartung *et al* 1989). Fibroblasts are connective tissue cells which secrete extracellular matrix rich in collagen, glycoaminoglycogens, glycoproteins and elastic fibres and so are involved in growth, healing and repair processes. They are small spindle shaped cells when inactive but when active they have branched cytoplasm with abundant rough endoplasmic reticulum around an elliptical enlarged nucleus and prominent nucleoli. The fibroblast can give rise to smooth muscle cells or bone or fat cells all of which are of mesodermal origin, and has the capbility to regress or re-differentiate.

1.3.3 The Cervix in Pregnancy

In early pregnancy, uterine muscle and fibrous elements hypertrophy and undergo hyperplasia. Cervical oedema and increased vascularity is evident. The length of the cervix remains unchanged in the first trimester, as does the smooth muscle content. Nor is there any consistent evidence of change in total elastin content, but it has been noted that elastin fibres are more numerous at the internal os (Leppert *et al* 1986). The existence of a distinct uterine "isthmus" was questioned after Danforth's work showed no anatomical or functional distinction between this and the uterine body (Danforth 1947). However the concept of the lower uterine segment (LUS), which establishes during the third trimester, has persisted and behaviour of this segment is clearly different to that of the uterine fundus. Is the isthmus or LUS merely a continuum between the uterus and cervix? As a transitional zone, this may possess similar properties to both regions above and below at different points in reproductive life.

Later studies revealed the cervical musculature to be slightly concentrated peripherally and more specifically, laterally in the supravaginal portion (Danforth 1954). The cervix is capable of contractile activity, though this is mild when compared to the abilities of the uterus. The purpose of the cervical smooth muscle

and elastin distribution may be to protect the uterine vessels and to aid restoration to normal postpartum function. Does this relative concentration of elastin and smooth muscle near the internal os suggest a sphincteric function, either in retaining the uterine contents antepartum, or restoring the cervix postpartum? (Leppert *et al* 1987)

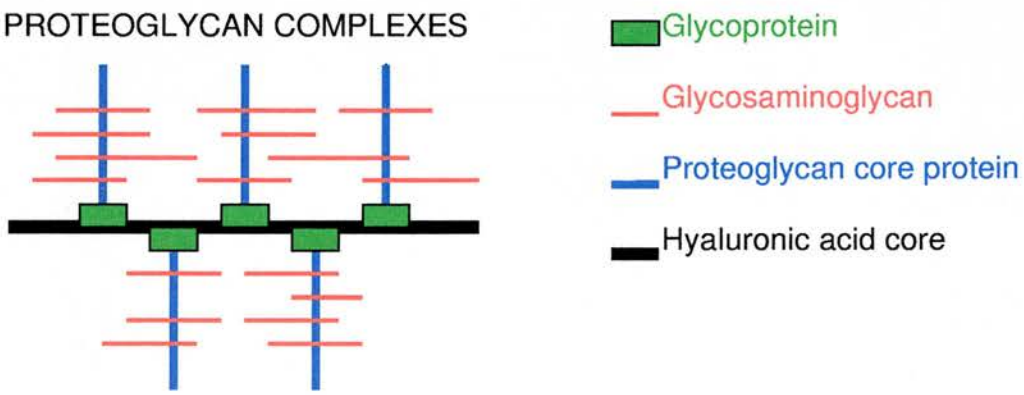
In late pregnancy, cervical smooth muscle cells appear hypertrophied and aligned in the vicinity of leukocytes, surrounded by collagen bundles in the presence of fibroblasts (Minamoto *et al* 1987). Apoptosis of smooth muscle cells has been observed in rat models (Leppert *et al* 1994) and may be involved in the initiation of extracellular matrix reorganisation. Fibroblasts do appear to undergo some changes in gestation at least in animal models. Bassett studied uterine, cervical and ligamental fibroblasts in the pregnant ewe and found a progressive enlargement of both fibroblast nuclei and cytoplasm with the nuclei being distinctly rounder as gestation advanced (Bassett 1959). These changes were accompanied by more sparse and loosely arranged collagen fibres, were not seen in other parts of the body and were also seen in other species (pig, cow, rabbit, guinea-pig). In the guinea –pig advanced gestation is associated with an increased number of activated cervical fibroblasts and a relative increase in mast cells and macrophages (Hegele-Hartung *et al* 1989).

In humans, there is no particular arrangement of type I and III collagens at any stage, but advancing gestation results in dissociation and branching of collagen fibrils into components with oedematous expansion of intervening spaces. The combination of collagen bundle reorganistion and apoptosis may, in concert, effect cervical ripening. A loosening of the collagen network is evident as early as the first trimester. In pregnancy and labour there is a large increase in the solubility of collagen and collagen breakdown products (the hydroxyproline containing peptides), relative to non-pregnant cervices (Kleissl *et al* 1978). Petersen et al demonstrated a lower cervical collagen concentration in women with cervical incompetence (as measured by hydroxyproline concentration and extractability), stressing the importance of the cervix in maintaining pregnancy in addition to its vital role in parturition (Petersen *et al* 1996). This study also demonstrated that pregnancy-related cervical changes do

not fully reverse with a gradual reduction in hydroxyproline concentrations following recurrent pregnancies. For collagen to retain tensile strength the fibres must exceed a critical length (20μm) and there must be strong chemical bonds between the collagen fibres and other matrix proteins. Throughout pregnancy there is a net loss of collagen fibre length, cross-linking and alignment and therefore loss of strength.

Studies on cervical collagen have used various methods from hydroxyproline content (both polymerised and depolymerised collagen) to picrosirius red staining (polymerised), with denominators of dry, wet or dry defatted tissue. Also different sites and types of biopsy obtained at different stages of pregnancy or labour have been compared. However, the following represents a summary of findings.

Extracellular matrix is composed of large molecules such as fibronectin, laminin and proteoglycan complexes. Proteoglycan complexes consist of various glycosaminoglycan (GAGs) side chains on core proteins, linked to a hyaluronic acid chain.



These complexes bind tightly, investing the collagen fibrils and providing rigidity. In general the longer the GAG chain the greater the binding affinity to collagen. The dominant GAGs in the cervix are chondroitin sulphate and dermatan sulphate. They contain iduronic acid, which binds very strongly helping to confer strength. Certain other glucuronic acid containing GAGs, such as heparan sulphate, bind less strongly leading to destabilisation of the organised fibril bundles, loss of mechanical strength and thus, enhanced compliance (Uldbjerg *et al* 1983b). This is represented by smaller widely scattered dissociated fibrils seen at term, compared to the non-pregnant state.

1.3.4 The Cervix in Labour

During active labour, the cervix is mechanically expanded by the downward force of uterine contractions on the fetal presenting part. Both the cervix and LUS become thinned and are passively dilated as the fetal head descends. For this to occur the cervix must have previously transformed to a softened, non-resistant state. Effacement is where the cervix shortens or thins and this may occur in either the latent or active phase of labour.

Biomechanically, the cervix is subject to cyclic stretch and relaxation, where greater cervical dilatation is achieved during contraction than by the end of contraction. The visco-elastic nature of the cervix (i.e. the mixed collagen and elastin components) contributes to this recoil effect, while allowing overall progressive dilatation.

There are many biochemical changes in the cervix peri-partum, the end result being a reduction in overall collagen content by 50-70% (Junqueira *et al* 1980; Uldbjerg *et al* 1983b), an increase in water content (from 80 to 86%), and a change in the proportions of different GAGs (Osmers *et al* 1993) with a relative increase in heparan sulphate (Danforth *et al* 1974; Uldbjerg *et al* 1983a; Ekman *et al* 1986). Recently it has been suggested that a large keratan sulphate proteoglycan may be involved in the matrix reorganisation at term, but this has not yet been fully characterised (Fischer *et al* 2001).

Hyaluronic acid (HA) content in the cervix also increases in labour, and as a hydrophilic compound may be partially responsible for the higher water content (Osmers *et al* 1993; El Maradny *et al* 1997). HA also induces neutrophil migration, collagenase synthesis, cytokine release (IL-1) and is secreted by fibroblasts, where there is some evidence of regulation by cytokines (IL-1, IL-8), hormones (progesterone), prostaglandins and lipopolysaccharide (see(El Maradny *et al* 1997) for review). HA is capable of producing cervical ripening in rabbit cervices, and is distributed throughout human cervical connective tissue. There appears to be a mutual interaction between IL-8 and HA in connective tissue remodelling. Rapid postpartum reduction of HA to normal levels has been shown previously and is thought to be as a result of progesterone induced depolymerisation regulated by the progesterone receptor (Tanaka *et al* 1994).

Human cervical connective tissue expresses both oestrogen and progesterone receptors, both of which are down-regulated as pregnancy advances (Stjernholm *et al* 1997). Progesterone inhibits IL-8 production and stimulates HA degradation, so with reduced PR effect at term the reverse may apply, encouraging ECM degradation. Human cervical studies have confirmed an increase in cervical IL-8 in and after labour but not with pre-labour cervical ripening (Sakamoto *et al* 2004; Tornblom *et al* 2005) so the role of IL-8 in cervical ECM reorganisation pre-labour remains uncertain, as does the distinction between labour or post-partum contribution to these changes. Additionally as no ripe cervices (Bishop score >8) were studied, then it could be argued that this data set is incomplete as IL-8 could be altered in this late stage of cervical ripening.

There is no doubt that these dramatic events in the extracellular matrix are an essential part of cervical ripening, and that significant activity in the connective tissue cells must be responsible to some degree. Studies in both guinea-pigs and humans concur on the cervical stromal appearances in labour. Both note a significant increase in mast cells, macrophages and in particular polymorphonuclear leukocytes, highly active fibroblasts and pericellular halos suggestive of collagenolysis (Junqueira *et al* 1980; Hegele-Hartung *et al* 1989; Montes *et al* 2002).

Outwith pregnancy fibroblast cells in the mucous layer of the human cervix stain for vimentin, but not α-smooth muscle actin or desmin. Those examined from intrapartum specimens display desmin positivity in association with myofibroblastic morphology as seen on electron microscopy, a finding peculiar to labour suggesting a specific phenotypic alteration for a particular function at that time (Montes *et al* 2002).

The fibroblast is undoubtedly central to this process as a source of collagen, GAGs and lytic enzymes in addition to the capability for phenotypic alteration, but there is more to consider. There are other contributory factors such as vascular permeability and its regulation (Cabrol *et al* 1990). What of migratory cells which do not constitutively form part of the connective tissue? There are many similarities to inflammatory events which will now be discussed.

1.4 INFLAMMATORY EVENTS IN THE CERVIX IN PREGNANCY

Infection has a long been identified as having a definite causative role in some cases of preterm labour, where there is an exaggerated inflammatory response leading to excess stimulation of cyclooxygenase pathways and cytokines. It is more recently that this theory has expanded to include parturition at all gestations, but the trigger without bacterial invasion will be different and the events appear attenuated. Pro-inflammatory cytokines now have a secure place in the pathophysiology of parturition in both term and preterm pregnancy. The extensive interaction between cytokines and prostaglandins in uterine, intrauterine and cervical tissues has been reviewed by Keelan et al (Keelan *et al* 2001).

Cervical ripening has been likened to an active inflammatory event culminating in extensive alteration within connective tissue (Junqueira *et al* 1980; Liggins 1981; Uldbjerg *et al* 1983a; Leppert 1995). There are several components; biochemical rearrangement of extracellular matrix, inflammatory reaction with enzymatic degradation, cervical smooth muscle relaxation and even apoptosis has been described (Leppert *et al* 1994).

The onset of cervical ripening occurs weeks before that of labour and cervical changes pre-exist even that. Vaginal washings display a significant increase in number of viable leukocytes and concentration of IL-8 towards term (Yamada *et al* 2002). Pro-inflammatory cytokines (IL-1, IL-8) increase in cervical stroma concurrent with cervical dilatation (Barclay *et al* 1993; Winkler *et al* 1998; Winkler *et al* 2003). In addition cervical ripening can be induced by PGE_2, NO and IL-8 in humans, and guinea-pigs (Uldbjerg *et al* 1983b; Rajabi *et al* 1988; Uldbjerg *et al* 1990; Calder *et al* 1993; Chwalisz *et al* 1994; Chwalisz *et al* 1997; Winkler *et al* 1999b; Winkler *et al* 1999a). There appears to be a temporal relationship between cytokine release, macrophage migration within uterine and cervical tissues and prostaglandin synthesis via COX-2 activity (Romero *et al* 1991; Bennett *et al* 1992; Allport *et al* 2001). Changes in macrophage numbers, activation and location have

been shown in mice and human uteri and may represent a shift from non-inflammatory to inflammatory response as gestation progresses (Thomson *et al* 1999; Ledingham *et al* 2001b; Yellon *et al* 2003).

There has been much work into the final "cervical ripening" seen in pregnancy but limited study into the cervix throughout pregnancy. For obvious reasons this is a difficult area to study but the preparatory phase of cervical remodelling that is likely to occur throughout gestation is an essential component to the events that follow. Late stage cervical ripening, cervical effacement, dilatation and the remarkable post partum recovery to a pre-pregnant state will all rely on gestation-dependent regulatory mediators.

1.4.1 Chemotaxis

Leukocyte recruitment occurs as a result of chemotactic stimuli, vasodilation and extravasation of immune cells, immune cell activation and MMP release. Chemokines are particular cytokines that induce chemotaxis (migration) and activation of leukocytes (Baggiolini 1998). As such, chemokines have an important role in inflammatory responses and pathological inflammatory disorders (arthritis, pulmonary fibrosis, hepatic fibrogenesis, multiple sclerosis). Fibroblasts have the ability to synthesize chemokines and therefore initiate leukocyte recruitment and inflammatory responses in both physiological and pathological situations. Thus the fibroblast is likely to play a key role in cervical remodelling. Chemokine synthesis is stimulated by bacterial products, pro-inflammatory cytokines, growth factors and hypoxia (Galindo *et al* 2001). With neutrophil chemotaxis there is an early requirement for cell adhesion, as has been demonstrated by Winkler and others, where an increased expression of adhesion molecules in the LUS/ cervix was shown in association with term and preterm labour (Ledingham *et al* 2001b; Winkler *et al* 2003). Expression was largely on vascular endothelial cells, but also on leukocytes, and *in vitro* studies revealed potential modulation by pro-inflammatory cytokines, PGE_2 and sex hormones.

Leukocyte chemotaxis occurs in several phases. Firstly, vascular endothelial cell adhesion molecules (VCAMs) are activated, weakly binding leukocyte cell adhesion molecules (CAMs) and causing these bound leukocytes to roll along the vessel wall. The high local concentration of cytokines causes the leukocyte to both shed its surface CAM, and upregulate integrin expression. Integrins bind much more strongly to the endothelial VCAM-1 or ICAM-1 and result in leukocyte arrest, adherence and flattening against the vessel wall. The chemokines present in the surrounding tissue stroma adhere to GAGs and attract the extravasating leukocytes along a chemokine gradient into the target tissue.

Chemokine receptors are seven-transmembrane-bound-G-coupled receptors (7-TMB). The antigen specific extracellular N-terminal domain is linked to the intracellular C-terminal domain by 7 hydrophobic α-helical transmembrane spanning regions. Activation is by binding to the extracellular domain, resulting in a conformational change coupled to intracellular signalling systems and 2nd messenger pathways. Inactive GDP is converted to active GTP via adenyl cyclase, phospholipase or tyrosine kinase pathways.

Liggins initiated the theory of the inflammatory reaction mechanism for cervical ripening (Liggins 1981). This proposes that vasodilatation, exudation, fibroblast proliferation and collagen remodelling; all typical of the inflammatory reaction occur in the cervix. Following leukocyte invasion, these cells degranulate, releasing matrix metalloproteinases which degrade the structural proteins of the extracellular matrix. Studies have observed a lack of collagen around neutrophils in the cervix peripartum, but whether this is due to collagen degradation or simply rearrangement is unclear (Junqueira *et al* 1980). Infiltration of the cervix by inflammatory cells may occur with an initial trigger of macrophage activation, release of cytokines (IL-1, Il-6, IL-8, PAF) and then enhancement of leukocyte recruitment. IL-8 is a known neutrophil chemoattractant and may have a synergistic effect with PGE_2 or NO, which are both vasodilators (Thomson *et al* 1997b; Kelly 2002). This would aid extravasation of these recruited cells from vessels into cervical stroma.

1.4.2 Migratory Cells

As early as 1980, there has been good evidence of cervical leukocyte infiltration in labour (Junqueira *et al* 1980). Surprisingly few studies since have adequately supported or refuted these findings. The original study compared non-pregnant cervical biopsies with those taken intrapartum and whilst reporting increased neutrophil numbers in labour compared to non-pregnant samples, also combined this with evidence of increased collagen depolymerisation. The biopsy method is not detailed but a thorough account of microscopy and electron microscopic findings are given. This does not allow assessment of gestation dependent changes outwith labour.

Bokstrom took cervical needle biopsies in early pregnancy, term pregnancy before labour and also in labour to study leukocytes subpopulations by immunohistochemistry, and showed T-lymphocyte predominance in the first trimester, compared to significantly increased densities of neutrophils and macrophages at term with no further significant increase in labour (Bokstrom *et al* 1997). The perivascular distribution of leukocytes was noted in both early and late gestation, but this added a gestation dependent alteration of leukocyte sub-types with no significant change seen in labour. Denison's study group was first trimester pregnancy and Shumaker punch biopsies of the cervix were compared after mifepristone administration. Immunolocalisation of leukocyte markers in untreated samples showed CD45 and neutrophil elastase to be mainly stromal whilst CD68 was negative. Mifepristone markedly increased all leukocyte markers in the stromal compartment, and epithelium in the case of CD68 (Denison *et al* 2000). T-cells were not distinguished in this study, but it confirms the presence of leukocytes early in gestation, and the phenomenon of leukocyte invasion after antiprogestin administration.

Stygar again confirmed an increased density of CD45 and CD68 immunopositive cells at term and post-partum, agreeing with Bokstrom (Stygar *et al* 2001). These

leukocytes and monocytes were largely seen perivascularly and in the subepithelial areas. Another comparison of non-pregnant, term and labouring cervices found increased intercellular cell adhesion molecule mRNA expression in labour in association with leukocytes, whereas VCAM mRNA only upregulated in pregnancy with no further increase with labour (Ledingham *et al* 2001b).

The table below summarises the differing timing of cervical samples taken in relevant studies.

Table 1.1

	NP	EP	TP	IP	PP
Junqueira 80					
Bokstrom 97					
Denison 00					
Stygar 01					
Ledingham 01					
Young 02					
Osman 03					
Sakamoto 04	cytokines				vag del
Stygar 02	MMPs				vag del
Sennstrom 00	cytokines				vag del

NP= late pregnancy, EP = early pregnancy, TP = term pregnancy, IP = intrapartum, PP = post partum.

Two studies compared term cervical biopsies taken at CS before or during labour (Young *et al* 2002; Osman *et al* 2003). Young immunolocalised proinflammatory cytokines, IL1-β and IL-6 to invading leukocyte populations, whereas IL-8 was distributed throughout the cervical stroma and epithelium in addition. In all cases the leukocyte density was increased in labour. Osman then further refined this by specifying the leukocyte subpopulations involved as neutrophils and monocyte types, identified by CD45, CD68 and neutrophil elastase immunohistochemistry. The distribution of these cells was largely stromal. These studies can then only relate

their findings to labour and no comparisons were made with early or non-pregnant cervical leukocyte populations. So Bokstrom and Stygar agree that this appears to be a gestational leukocyte invasion prelabour, whereas most of the other studies conclude that this leukocyte recruitment occurs only in labour.

In assessing the cervix throughout pregnancy and labour, one ideally needs to study cervical tissue from all stages; non-pregnant, first trimester, mid-trimester, term pregnancy before labour (ripe and unripe cervices), in labour and post-partum. Clearly there are ethical issues that have made this area of study difficult, but one must carefully compare the evidence available with these distinctions in mind.

Even these groups cannot be equally compared as TP groups are not always described as ripe and unripe cervices. This is an important differentiation and intrapartum groups are not always comparable dependent on the stage of labour and reason for caesarean section. Some groups have endeavoured to take samples specifically after vaginal delivery as this is the physiological outcome (Stygar *et al* 2001; Stygar *et al* 2002; Sennstrom *et al* 2003; Sakamoto *et al* 2005), but many groups only take samples in those women delivered electively by CS, which may be relatively distant from cervical ripening, or those delivered in labour by CS (Young *et al* 2002; Osman *et al* 2003). Some effort has been made to exclude dysfunctional labour and infection but this is by no means consistent across study populations. Most of this work has only informed on leukocyte population changes in or after labour, omitting cervical ripening pre-labour.

Recent localisation studies demonstrate cervical CD45+ leukocyte numbers to be greater in the subepithelium than deep stromal layer outwith pregnancy, in early and late pregnancy and after labour (Sakamoto *et al* 2005). Vaginal delivery is associated with increased macrophage and leukocyte populations in both the subepithelium and deep stromal layer in association with high IL-8 levels (Sakamoto *et al* 2004). As these specimens were obtained postpartum, a distinction between labour related or post-partum changes cannot be made. Prior studies had not determined pre-labour leukocyte population alterations in the ripe and unripe cervix as these groups had not

been specified. Sakamoto's work attempts this by dividing the term pre-labour group into unripe, ripening and ripe cervices based on a Bishop score (BS) of ≤ 4, >4 or >8 (Sakamoto *et al* 2005). It appears that macrophage but not granulocyte presence is associated with pre-labour cervical ripening. Crucially with a BS > 4, a rise in macrophage presence was shown in both the subepithelium and deep stroma but as there were no samples in the ripe cervix group (BS>8), further conclusions cannot be drawn.

Early pregnancy appears to be associated with a specific picture of leukocyte distribution as described by Junqueira but no quality assessment of this has been performed since. The dramatic leukocyte infiltration seen is certainly a late event and appears to be associated with labour, but whether this is part of labour initiation, established labour, or a post-partum phenomenon has yet to be established.

Animal studies have been of little help with this issue as most animal models do not appear to follow the same pattern of immune cell recruitment. Rat models certainly show cervical eosinophil invasion at term and this appears to be under hormone control (Luque *et al* 1998), but then there are many differences between the rat and human model, not least the luteolytic progesterone withdrawal with initiation of labour, and so this is not informative.

Mouse models appeared to confirm macrophage migration to the cervix prior to labour as has been seen in humans (Yellon *et al* 2003). In a recent paper (published after submission) Mahendroo's 5alpha-reductase null mouse model concludes neutrophils are not involved in cervical ripening but in post partum remodelling (Timmons *et al* 2006). Cervical samples of wild-type mice show no increase in leukocyte numbers or activity, or cytokine expression or release until after delivery, when the predominant cell migration appears to be the macrophage and the eosinophil. Regulated by a progesterone fall there is a stromal recruitment of neutrophils from the subepithelium pre-labour and this is not seen in 5alpha-reductase null mice (Timmons *et al* 2006). However there is no increased neutrophil activity until after delivery, suggesting no role in pre-delivery ECM degradation, but

rather preparation for postnatal remodelling and repair. Although this parallels Sakamoto's findings (Sakamoto *et al* 2005), there are fundamental differences from the human model such as the longstanding acknowledgement of differing systemic and local tissue progesterone control between rodents and humans. Also whilst this refutes a role for these cells in pre-labour cervical ripening, neither of these groups have assessed intrapartum events. The responsibility of ECM degradation pre-delivery then falls again to the resident stromal cell if there is little leukocyte activation.

Monocyte type cells are a known source of proinflammatory cytokines, which have increase in labour. Guinea pig, rabbit and sheep models all appear to show some cervical immune cell trafficking. For example an electron microscopy description of guinea-pig cervices throughout gestation reported fibroblasts as the main stromal cellular component, with few leukocytes and densely packed collagen fibres with little amorphous ground substance between (Hegele-Hartung *et al* 1989). In late pregnancy an increase in leukocyte numbers, hypertrophied smooth muscle cells and increased amorphous ground substance with dissociated collagen fibrils in association with active fibroblasts, mast cells and macrophages was evident. Term cervices displayed more marked connective tissue disassociation, oedema, fibroblast activity and a predominance of neutrophils. Of note these findings were then replicated after administration of a progesterone antagonist in late gestation. Significantly, the highly active fibroblast appeared with a halo of reduced collagen surrounding it, as had been previously noted with leukocytes (Junqueira *et al* 1980). This halo reflects the absence of collagen fibrils and is presumed to be a result of collagenolytic enzymes. It can be postulated that the association between macrophage presence and timely increase in human cervical stromal cytokine release would place the macrophage in a central role in the final pre-labour cervical remodelling phase (Winkler *et al* 1998; Sennstrom *et al* 2000; Young *et al* 2002; Sakamoto *et al* 2004).

The association of cervical leukocyte migration with labour is clear yet the evidence for pre-labour recruitment is less so. This raises the question of whether these cells

are required for late stage cervical ripening, the ongoing cervical changes throughout pregnancy or whether their presence is solely required for, or even a result of, active labour. It appears that the appropriate studies have not yet been performed to clarify this issue as rather than comparing pre and post labour, one would need to compare ripe and unripe cervices outwith labour. Recruitment in this particular group can be extremely difficult for ethical reasons.

Less invasive methods of assessing cervical ripening may be the way forward in this area, but this will not provide all the information available from tissue samples. Outwith clinical practice, other methods have been used to assess cervical state. Fluorescence spectroscopy can inform molecular and physical structure and has been used to examine collagen content in various tissues. Garfield et al have used this technology in the form of a collascope to assess cervical tissue changes in pregnancy in both rats and humans, the advantage being the ability to perform longitudinal studies in the same subjects. Light-induced autofluorescence (LIF) in the cervix is thought to originate from pyridinoline, a major crosslink in collagen fibril structure, and was seen in rats to reduce in late gestation and after treatment with antiprogesterone (Garfield *et al* 2001). These findings were corroborated by parallel cervical resistance studies and electron microscopy and the changes spontaneously reversed. Human studies have shown a similar decline in the last 15 weeks of pregnancy with slow postpartum recovery, and may prove clinically useful in prediction of preterm labour (Maul *et al* 2003). The non-invasive nature of LIF may aid future investigations in cervical function in addition to potential benefits in labour management.

1.4.3 Matrix Metalloproteinases

MMPs are zinc-dependent degradative enzymes secreted by both connective tissue cells and leukocytes as pro-MMPs, which are then activated by proteinases such as plasmin or indeed by other MMPs (Hulboy *et al* 1997). Endogenous inhibitors tissue inhibitors of metalloproteinases (TIMPs) and α-macroglobulins form complexes

with activated MMPs to inactivate them. ECM maintenance is reliant on the balance between both MMPs and their inhibitors and matrix synthesis and disposition. Control of their production is carefully timed throughout pregnancy and parturition and regulated by cytokines, growth factors and hormones (Hulboy *et al* 1997). Immediately prelabour the ripening process seems to accelerate with even more proteolytic activity associated with an inflammatory cell infiltrate (Knudsen *et al* 1997).

The main role of MMPs is tissue remodelling by processing matrix proteins and collagens. This is evident in many physiological inflammatory processes i.e. menstruation, ovulation and wound healing. Pathological disease processes such as cancer and fibrosis also require MMP activity. The same process may aid cell migration by degradation of basement membrane constituent collagen type IV, and this is particularly relevant in human reproductive tissues where migratory cells accumulate at various stages within the reproductive cycle. Specific to the cervix there is a leukocyte extravasation associated with labour (Junqueira *et al* 1980; Stygar *et al* 2001; Young *et al* 2002; Osman *et al* 2003).

Classification of MMPs can be broadly divided into four categories:

1) interstitial collagenases (MMP-1, -8, -13); degrade intact collagen
2) gelatinases (MMP-2,-9); degrade proteoglycans, denatured collagen I and III, collagen type IV, elastin, fibronectin
3) stromelysins (MMP-3,-7,-10,-11)
4) membrane-type MMPs (-14,-15,-16,-17).

The interstitial collagens (type I-III) are initially cleaved by interstitial collagenases, predominantly MMP-8, MMP-1 and MMP-13. Then, after further denaturation to gelatin, gelatinase (MMP-2 and MMP-9) activity results in small peptides which are finally degraded by proteolytic enzymes (Hulboy *et al* 1997). Various studies have reported increased levels of these enzymes in the cervix and lower uterine segment in late pregnancy and labour (Junqueira *et al* 1980; Kitamura *et al* 1980; Rath *et al*

1987; Rajabi *et al* 1988; Osmers *et al* 1990; Winkler *et al* 1999a; Stygar *et al* 2002; Sennstrom *et al* 2003).

MMP-1 or fibroblast collagenase is secreted by both stromal cells and monocytes, and degrades collagen I, II, III. MMP-1 degrades collagen type III 15-fold faster than type I. MMP-8 or neutrophil collagenase is secreted by both neutrophils and cytokine-activated fibroblasts and responsible for breakdown of fibrillar collagens. MMP-8 is particularly efficient at collagen type I cleavage and also has substrate specificity for gelatins and non-matrix proteins (Horwitz *et al* 1977; Hasty *et al* 1987).

The substrates of MMP-2 include fibrillar collagen, collagen IV, elastin and fibronectin (Hulboy *et al* 1997). MMP-2 also activates MMP-1, -9 and -13. Of particular interest is the capability of basement membrane degradation by MMP-2 as this has been associated with pathological events involving monocytes and specifically in tumour invasion (Sato *et al* 1994; Yoshizaki *et al* 2002). A similar physiological process may apply in cervical ECM proteolysis during pregnancy. A parallel can be drawn with the endometrium in menstruation where MMP-2 and MMP-14 are upregulated with progesterone withdrawal (Zhang *et al* 2000).

MMP-9 produced by macrophages, neutrophils and cytokine stimulated fibroblasts catalyses collagen types IV and V and elastin degradation (Winkler *et al* 1999b; Winkler *et al* 1999a).

MMPs-1,-2,-8 and -9 have been studied in the human cervix and lower uterine segment (Ledingham *et al* 1999a; Winkler *et al* 1999a; Denison *et al* 2000; Stygar *et al* 2002; Sennstrom *et al* 2003). In the human cervix, neutrophils predominantly produce MMP-8 and MMP-9, whereas MMP-1 is released by cervical stromal fibroblasts both in early pregnancy and outwith (Yoshida *et al* 2001). Stygar et al demonstrated by immunohistochemistry and reverse transcription-polymersase chain reaction (RT-PCR) that in vivo, cervical MMP-9 was observed exclusively in invading leukocytes perivascularly, and that this was up-regulated peripartum

(Stygar *et al* 2002). The same study identified cervical stromal fibroblast cells and smooth muscle cells as the main source of MMP-2 and this is supported by work with cervical explants and isolated cervical fibroblasts from both non-pregnant and first trimester samples (Ledingham *et al* 1999a). Denison et al immunolocalised MMPs-1,-2,-8 and -9 in the first trimester cervix with increased stromal immunostaining for all except MMP-2 after mifepristone treatment in vivo (Denison *et al* 2000). Cervical MMP-2 and MMP-9 mRNA expression is up-regulated in pregnancy at term, but there is no further increase seen after labour (Stygar *et al* 2002).

Watari demonstrated that cultured cervical smooth muscle cells express and secrete MMP-1, -9 and -13 but it must be noted that these cells were derived outwith pregnancy (Watari *et al* 1999). Combined, these studies suggest that MMPs have multiple origins and whilst leukocyte infiltration may provide abundant supplies of some MMPs, there is a role for resident cells in cervical MMP synthesis and regulation. It is the interaction and individual activation of these differing cell types that may hold the key to cervical changes in pregnancy, and it is this that makes it extremely difficult to study *in vitro.*

Many studies have used the lower uterine segment as a proxy for the cervix, in view of the ethical difficulties associated with obtaining cervical tissue in advanced pregnancy or in labour. Rajabi did just this to confirm comparison baseline levels of collagenase in cervical and LUS tissue outwith labour (Rajabi *et al* 1988). Analysis of LUS biopsies in labour demonstrated a thirteen-fold increase in active and latent collagenase levels but a specific collagenase or source was not identified, and there were no cervical samples taken in labour. A similar study in 1999 revealed an increase in both MMP-8 and MMP-9 in the LUS with increased cervical dilatation and duration of labour (Winkler *et al* 1999a) and associated with increased IL-8 concentrations and neutrophil counts (Winkler *et al* 1999b).

Although cervical fibroblasts secrete MMP-1, preferentially degrading collagen III, a cervical up-regulation of this particular MMP has not been shown in parturition

(Osmers *et al* 1992). MMP-2, MMP-8 and MMP-9 are shown to increase throughout gestation and even more so at the time of parturition, as the cervical collagen content decreases (Uldbjerg *et al* 1983b; Rajabi *et al* 1988; Osmers *et al* 1992; Stygar *et al* 2002; Sennstrom *et al* 2003). Cervical MMP-8 and MMP-9 levels increase with cervical dilatation and neutrophils counts yet no distinction can be made between labour effects and those specific to cervical ripening due to the timing and grouping of samples (Sennstrom *et al* 2003). MMP-8 may breakdown collagen whilst MMP-2 and MMP-9 also assists in leukocyte stromal extravasation by degrading basement membrane.

MMPs exist either as free molecules, in complexes with TIMPs or complexed to α_2-macroglobulin, both of which are inhibitory; it is the balance between these complexes that influences MMP activity and function. Regulation can occur at transcription, post-transcription or post-translation as a result of pro-enzyme cleavage or TIMP-binding. Tissue inhibitors of metalloproteinases (TIMPs- 1, 2 and 4) modulate MMP activity are also present in the cervix and increase with pregnancy (Ledingham *et al* 1999a; Denison *et al* 2000; Sennstrom *et al* 2003). Alpa$_2$-macroglobulin inhibits MMPs by forming complexes inactivating them and is not upregulated with parturition to the same extent as MMPs themselves. For example, LUS collagenase concentration was 23-fold in labour compared to term whereas its inhibitors increased only 2-fold in labour (Woessner 1991; Rechberger *et al* 1993). MMP-2 and MT-MMPs are constitutive whereas most others require stimuli to promote transcription. Such stimuli are cytokines, progesterone, oestrogen, glucocorticoids and nitric oxide.

The regulation of MMPs is multifaceted but cytokines appear to induce production and activation whereas progesterone is suppressive in cervical studies. Rabbit and human cervical fibroblasts produce proMMP-9, proMMP-1 and proMMP-3 with cytokine (IL-1 or TNFα) stimulus and these effects are inhibited by progesterone (Ito *et al* 1991; Sato *et al* 1991; Sato *et al* 1996; Imada *et al* 1997). Similarly in the human cervix in-vivo first trimester treatment with the antigestogen mifepristone revealed an up-regulation in immunostaining for MMPs-1, -8 and -9, neutrophil

elastase, CD45 (leukocyte common antigen) and CD68 monocyte marker (Denison *et al* 2000). Untreated cervices showed a down-regulation of MMP-9 in early pregnancy perhaps representing the protective effect of progesterone at this gestation (Ledingham *et al* 1999a).

Human non pregnant cervical smooth muscle cells secrete increased MMP-1, MMP-3 and MMP-9 in response to TNFα or IL-1 and IL-1 induced MMP-1 secretion has been shown in early-pregnancy-derived cervical fibroblasts (Watari *et al* 1999; Yoshida *et al* 2002). Both Osman and Young demonstrate increased cervical IL-1β, IL-6 and IL-8 localised to leukocytes in association with labour providing a mechanism for labour associated MMP upregulation (Young *et al* 2002; Osman *et al* 2003). Cultured cervical fibroblast IL-8 production is induced by cyclical mechanical stretch designed to mimic the effects of pregnancy and labour (Takemura *et al* 2004). Wang et al suggest that induction of MMP-14 and MMP-2 expression by cyclical stretch is via TNFα and although this study was in cultured human umbilical vein endothelial cells, it presents supportive comparisons to the body of evidence within the cervix (Wang *et al* 2003).

$PGF_{2\alpha}$, NO and mechanical stretch all augment MMP-1 secretion from cultured human cervical fibroblasts (Yoshida *et al* 2001; Yoshida *et al* 2002) and although cervical MMP-2 and MMP-9 secretion was not altered with in-vivo NO donor treatment, other MMPs may be influenced (Ledingham *et al* 1999b). Cervical NO synthase isoforms, localised to the endothelium and leukocytes, are elevated in early pregnancy with a further increase at term prelabour suggesting a role in the gradual cervical changes seen throughout pregnancy rather than late cervical ripening (Ledingham *et al* 2000). Cervical prostaglandin degradation is certainly decreased as a late event in association with labour whereas COX-2 is unchanged, suggesting prostaglandin effects are elevated at this time (Tornblom *et al* 2004). Progesterone, NO, stretch, cytokines and prostaglandins all influence MMP regulation and based on current evidence this chronological sequence of mediation effects appears likely.

The interdependency of MMP function complex as each MMP provides substrate for another in the stepwise process of ECM degradation. MMP-1 specific evidence is based only on in-vitro findings. MMP-2 is thus far the most likely candidate produced by cervical stromal cells to influence cervical remodelling in pregnancy. MMP-2 activity is regulated in part by monocyte MMP-14. MMP-14 is a membrane-type MMP (MT1-MMP) and activates MMP-2 though a prostaglandin-cAMP pathway as has been established in activated monocytes (Shankavaram *et al* 2001). MMP-14 is membrane-bound and has wide substrate specificity for collagen types I, II and III, elastin, fibronectin, gelatin, laminin and other proteoglycans. In addition MMP-14 activates the inactive proform of MMP-13. Expression of MMP-14 has been confirmed in activated fibroblasts, monocytes, endothelial cells, decidua, fetal membranes, osteoclasts, trophoblast and cancer cell membranes (Fortunato *et al* 1998; Nakano *et al* 2001). In the human cervix the presence of MMP-14 has only been demonstrated in squamous cell cancer but has not been evaluated in the context of pregnancy or cervical ripening (Sheu *et al* 2003). However, MMP-14 is also associated with leukocytes and the activation of proMMP-8 (Holopainen *et al* 2003). As both MMP-2 and MMP-8 levels are increased in pregnancy and labour then MMP-14, as a mediator of at least these two MMPs, would be expected to be present in the human cervix and up-regulated in pregnancy or labour.

In addition to ECM maintenance MMPs also have the capability to alter the cellular phenotype of tissues, thereby influencing proliferative, differentiating and apoptotic functions (Hulboy *et al* 1997). It is pertinent to know whether the phenotype of any of these cervical or migratory cells change during pregnancy. Indeed there has been a myofibroblast-type differentiation seen within the human cervix in labour, where these submucosal fibroblasts demonstrate desmin positivity and associated high activity as shown by electron microscopy (Montes *et al* 2002). Active fibroblasts have multiple potential secretory activities and could be central in orchestrating cervical remodelling seen throughout pregnancy, labour and post-partum.

1.4.4 Prostaglandins

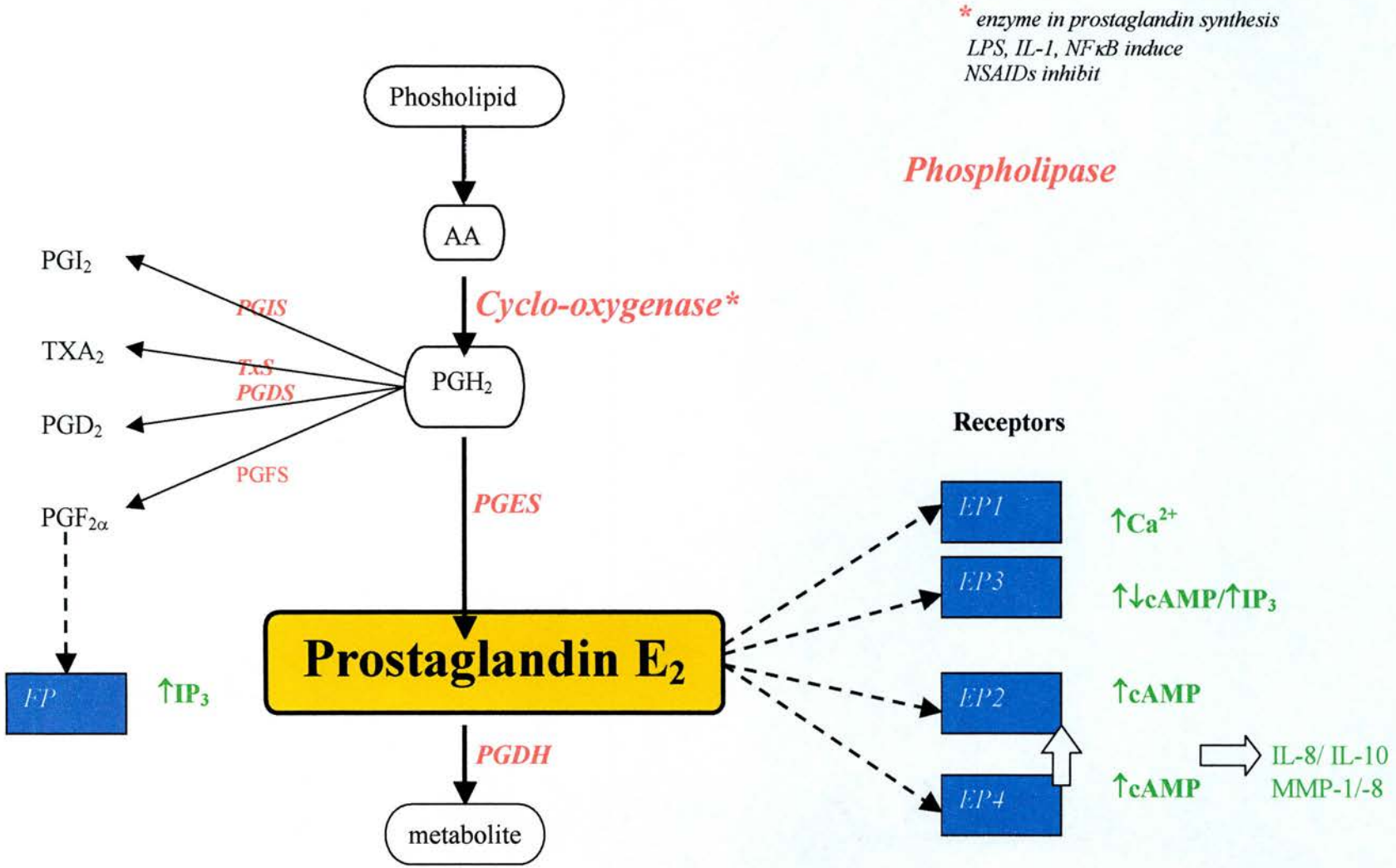
Prostaglandins are eicosanoids, of which those believed to be most important in human labour are of the 2 series, PGE_2 and $PGF_{2\alpha}$. They are derived from the cyclooxygenase (COX) metabolism pathway of arachidonic acid, obtained from deesterified membrane phospholipids. PGs are locally acting paracrine regulators, rapidly catabolised from the circulation in the lung, and also locally metabolised by degradative enzymes, and so require prompt tissue response for any effect (Moore 1985; Kelly *et al* 1992). PGs are produced by many tissues, but notably by amnion, chorion, decidua, placenta, cervix, leukocytes and platelets. There is no storage mechanism so they are produced and released as required, and act through specific G-protein coupled receptors (Geirsson *et al* 1990).

Prostaglandins induce many effects throughout all systems including pro/anticoagulant properties, immunomodulation, vasoconstriction/dilation and bronchoconstriction/dilation, hence their potency and potential for unwanted side effects as well as desired attributes.

There are five groups of prostaglandins. Those involved in pregnancy are PGE_2 and $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane, but in labour only the first two have identified roles. PGE_2 levels are known to increase in late pregnancy and early labour whereas $PGF_{2\alpha}$ rises in established labour (Moore 1985). See Figure 1.4

Many sources of prostaglandins have been identified in the cervix (Ellwood *et al* 1980), the uterus, placenta and fetal membranes. The amnion is particularly capable of PGE_2 synthesis (Keirse 1979; Mitchell 1987a; b) and it has been shown that amniotic fluid prostaglandin levels gradually increase towards term with a sharper rise in labour and as labour progresses (Steinborn *et al* 1995; Reece *et al* 1996; Gibb 1998). This is now known to be as a result of increased synthesis, rather than reduced catabolism. As prostaglandins and their controlling enzymes (phospholipases, cyclooxygenases, and prostaglandin dehydrogenase) are fairly ubiquitous is has

Figure 1.4 *Prostaglandin metabolism*



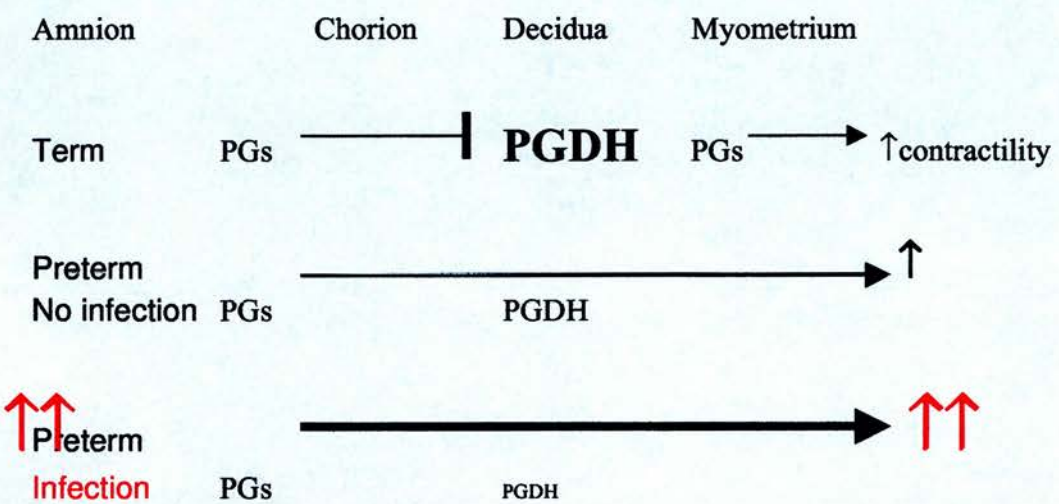
proved difficult to localise production sites, although high prostaglandin levels have been reported in the amnion, decidua ($PGF_{2\alpha}$) and myometrium.

There are two cyclooxygenase enzymes, COX-1, a constitutive enzyme and COX-2, an inducible enzyme that increases in labour (Bennett *et al* 1992; McLaren *et al* 2000; Whittle *et al* 2001). Particularly high levels of the prostaglandin inactivating enzyme PGDH have been found in the chorion and trophoblast. Although placental levels do not fall in labour, chorionic PGDH expression was seen to be lower in both term and preterm labour compared to non labour (Whittle *et al* 2001). Another theory is that with varying membranous PGDH distribution there may be regional variation, most pertinently covering the internal cervical os, where the prostaglandin can escape catabolism and traverse the membranes in order to effect cervical ripening and myometrial activity (van Meir *et al* 1997; Van Meir *et al* 1997; Challis *et al* 1999). See Figure 1.5

Intrauterine prostaglandin levels, synthesis and catabolism have been extensively studied with the consensus being that prostaglandin production is increased with term and preterm labour (Mitchell 1987a; Olson *et al* 1992; Gibb 1998). Pro-inflammatory cytokines (IL-1, TNF-α) appear to enhance prostaglandin production in many tissues including amnion, chorion, decidua and myometrium via the inducible COX-2 pathway (Keelan *et al* 2001). The inflammation-associated transcription factor NF-κB, activated by pro-inflammatory cytokines, is expressed constitutively in amnion at term. This not only increases COX-2 expression but also appears to negatively interact with the progesterone receptor facilitating 'functional progesterone withdrawal' (Allport *et al* 2001). This combination of events then triggers PG synthesis and activation of CAPs leading to labour. The possibility of prostaglandin regulation of cytokines in an auto-regulatory loop has been suggested as PGE_2 stimulates cervical IL-8 release, but supportive data in the placenta and membranes is lacking (Denison *et al* 1999).

Cervical biopsies taken after both at term and preterm delivery, with and without labour confirm a reduced expression of PGDH when labour had occurred, suggesting

Figure 1.5: *Altered PGDH production in term and preterm labour*



decreased prostaglandin degradation in association with parturition (Tornblom *et al* 2004).

Glucocorticoids increase amniotic COX-2 expression and decrease PGDH in the chorion thereby promoting prostaglandin activity (Gibb 1998). It is possible that PGDH expression may be varied in some areas by inflammatory cytokines as a result of infection, and in others by progesterone and cortisol competitively binding to their steroid receptors. Cheng showed a reduction in endometrial PGDH with concurrent increased PGE_2 following treatment with the antiprogesterone RU486 (Cheng *et al* 1993). The PGE_2 was localised to small blood vessels and this may aid leukocyte infiltration, supporting the hypothesis of synergism between IL-8 and PGE_2, proposed by Colditz in rabbit skin (Colditz 1990). Immunostaining later confirmed the perivascular location of IL-8 in non-pregnant endometrium (Critchley *et al* 1994) consistent with this proposed role.

PGE_2 is associated with a time-limited enzymatic collagen degradation, increased protein synthesis and increased hyaluronic acid concentration (Rath *et al* 1987). PGE_2 increases newly synthesized GAGs in cultured cervical fibroblasts in a dose-dependent manner (Carbonne *et al* 2000). Whether PGE_2 exerts collagenolytic effects directly is questionable as COX-2 inhibitors do not prevent these, and PGE_2 is likely act in concert with NO, cytokines and leukocyte recruitment.

Prostaglandin receptors are seven-transmembrane-G-coupled receptors divided into five groups, specific to their prostaglandin. The EP group are specific to PGE and are further subdivided into four isoforms; EP1 and EP3 stimulate the adenyl cyclase system causing muscle relaxation, whereas EP2 and EP4 are inhibitory and cause contraction (Arias 2000). EP2 and EP4 are present in cervical fibroblasts but only EP4 mediates PGE_2 stimulated GAG synthesis in a PKA-independent pathway (Schmitz *et al* 2001).

Both PGE and $PGF_{2\alpha}$ cause myometrial contractility whilst PGE2, in addition, has specific effects on cervical ripening. Cervical PGE production increases at term and

in labour and administration of PGE results in cervical changes akin to those seen physiologically. Prostaglandins, which are known to increase physiologically at this stage and are also effective therapeutically, are only one of several factors involved in initiation of this process.

The antiprogestin mifepristone (Radestad *et al* 1990), oestrogen, cytokines and other inflammatory mediators such as nitric oxide donors (Thomson *et al* 1998) are all implicated in the cervical ripening process but the exact mechanisms of interaction remain unclear. Prostaglandin E_2 reproduces cervical ripening in vivo and in vitro. However physiological or antiprogestin-induced cervical ripening is not suppressed by indomethacin or specific COX-2 inhibitors (Chwalisz 1994; Shi *et al* 2000). NOS and COX inhibitors combined are more effective in suppressing ripening than each alone, suggesting that these two inducible systems function in parallel (Shi *et al* 2000). Therefore, PGE_2 cannot be the only pivotal pathway involved. One must then ask how critical is the role of prostaglandins? And in clinical practice despite the clear advantage of PGE_2 use in induction of labour, this strategy has not been sufficient to reduce the growing caesarean section rate.

1.4.5 Steroid Hormones

In humans, there is no systemic reduction in progesterone in association with late gestation or labour (Anderson *et al* 1985). In any event, cervical ripening is a prolonged preparatory process occurring before the initiation of labour and myometrial activity. In fact progesterone levels do not fall until after the placenta is delivered and the cervix begins to remodel to a more rigid non-pregnant state (Garfield *et al* 1998). Yet antiprogestins do produce cervical ripening, but by what mechanism? It is therefore possible that local progesterone or receptor changes (Stjernholm *et al* 1997) may achieve a progesterone withdrawal effect to allow cervical changes as seen when antiprogesterones are administered.

Progesterone withdrawal is fundamental in parturition in small mammal physiology, either by luteolysis or reduced progesterone action. There may be local changes in progesterone metabolism or receptor activity in humans that mirror this. Progesterone can inhibit cytokine release, suppress iNOS / COX-2 expression, leukocyte migration and suppress MMPs (Ito *et al* 1994; Buhimschi *et al* 1996; Denison *et al* 1999). These effects would inhibit cervical ripening, and provide a mechanism of action for antiprogestin cervical ripening, as seen in animals and humans.

Progesterone withdrawal (by antiprogestin administration) effects cervical ripening in humans (Frydman *et al* 1988) and results in increased myometrial responsiveness. Similar structural changes (collagen fibre dissolution, oedema, influx) as seen in humans were described by Hegele-Hartung after studying progesterone antagonist exposed guinea-pig cervices by electron microscopy (Hegele-Hartung *et al* 1989). However progesterone agonists do not prevent antiprogestin-induced cervical ripening, so there must be more than PR modulation in the stimulation of these pathways (Shi *et al* 2000). Shi et al propose that progesterone and NO interact to maintain pregnancy and showed that antiprogestins in combination with NOS inhibitors stimulate labour in rats (Shi *et al* 2003).

E2 (17β oestradiol) increases myometrial activity in women and is associated with cervical ripening, collagenolysis in guinea pigs and eosinophilic invasion in rats (Pinto *et al* 1964; Rajabi *et al* 1991b; Luque *et al* 1998). P4 inhibits E2 stimulated collagen degradation *in vitro* (Rajabi *et al* 1991a). Plasma DHEAS increased during cervical ripening and increases procollagenase production in rabbit cervical fibroblasts (Sakyo *et al* 1987), although neither E2 or DHEAS affected collagen synthesis.

Cervical ripening in women is seen concurrently with a down-regulation of oestrogen receptors. This altered ER:PR ratio is associated with proteoglycan change and increased collagen solubility. Oestrogen and progesterone receptors are present in human cervical tissue. Although mRNA is unchanged, human cervical ER and PR

concentrations are reduced postpartum compared to term pregnant unripe cervices (Stjernholm *et al* 1997). The same also applied to IGFBP-1. However IGFBP-1 mRNA increased four-fold from the non-pregnant state to term, so is there an interaction between growth factor pathways and steroid receptors or do growth factors stimulate MMPs? This may serve as a local receptor-mediated progesterone withdrawal. PGE_2 treatment of human cervices at term did not alter cervical ER/ PR/ IGF-1 concentrations or mRNA expression when compared with spontaneous ripening, but mifepristone appeared to increase both ER and PR concentration so perhaps differing mechanisms are at work (Stjernholm *et al* 1999). This would support the role of prostaglandins and IGF-1 in spontaneous ripening, but does not support the concept of receptor-mediated progesterone withdrawal. More recently differing PR isoforms have been identified. PR-A and PR-B exist in myometrium and have opposing effects. PR-A is a dominant repressor of PR-B and labour is associated with raised PR-A in myometrium, with an increased PR-A: PR-B ratio (Winkler *et al* 2002). So, again this may come back to receptor mediation as more is discovered about individual isoforms, their expression, regulation and function.

1.4.6 Cytokines

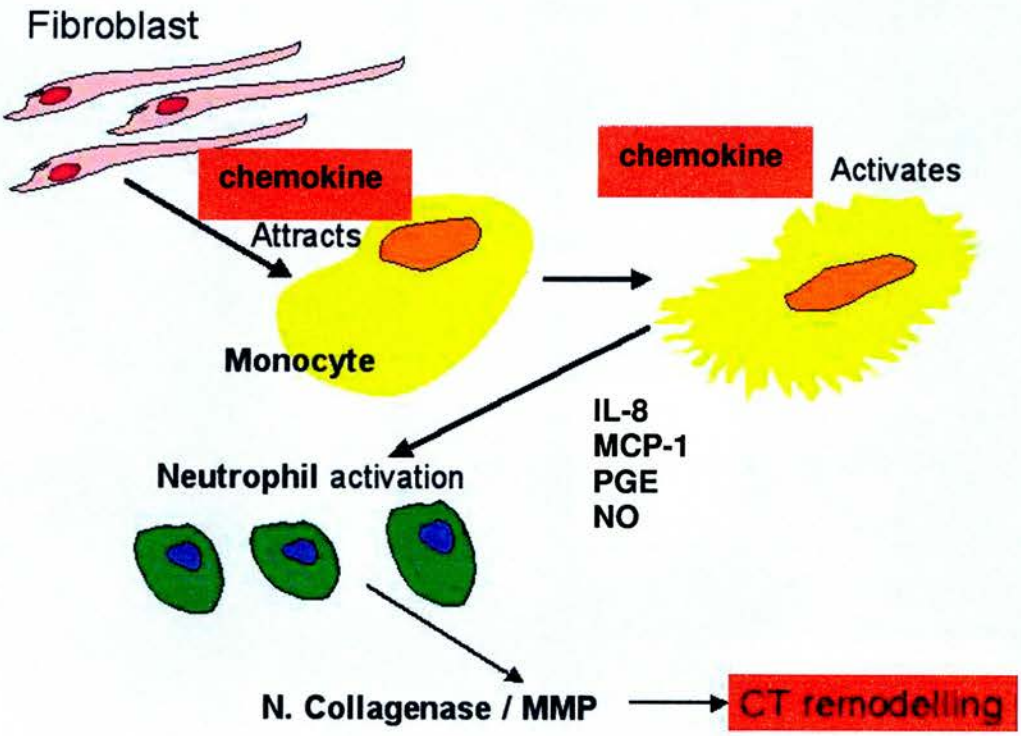
Leukocytes appear to have a major role in cervical changes in pregnancy, akin to an inflammatory response (Liggins 1981) perhaps orchestrated by the fibroblast with increased local prostaglandins, a marked inflammatory neutrophil infiltration (Junqueira *et al* 1980), increased vascularity and stromal oedema (see Figure 1.6). The reduced collagen content seen in late pregnancy is a result of both proteoglycan constituent variation and collagen breakdown from enzymatic degradation.

Cytokines are small secreted soluble proteins or glycoproteins which bind to specific cell surface receptors, couple to intracellular signalling systems and act via second messenger pathways. Cytokines can modulate cell function at nano-picomolar concentrations through humoral regulation or autocrine and paracrine routes. They are synthesized locally on demand and have short half-lives limiting the temporal

effects. Examples are interleukins, chemokines, interferons, growth factors and colony stimulating factors. Effects are achieved through altered gene expression, binding with membrane receptors (IL-8) or ECM complexes, receptor modulation or enzymatic cleavage to an active component. Cytokines are both pleiotropic and redundant and are often produced in cascades, creating complex interactive mechanisms of action. There are vast numbers of cytokines categorised by their producing cells, target cells, receptors or function of immune, inflammatory or haematopoietic regulation (see http://www.copewithcytokines.de/). All methods of categorisation are problematic in view of the degree of overlap and cross-talk between cytokine groups and receptors. An extremely select summary of a few cytokines described in the human cervix, or cervical stromal cell, specific to pregnancy and labour follows.

Pro-inflammatory cytokines stimulate immune cell proliferation and differentiation and include IL-1(α and β ligands) and TNF-α, produced by many cell types. TNF-α induces neutrophil chemotaxis, collagenase release, cytolysis, angiogenesis, and prostaglandin release. IL-1 stimulates T-helper cells, activates neutrophils and induces ACTH release and immunoglobulin production. IL-6 is immunomodulatory and acts on monocytes, macrophages and lymphocytes as a physiological regulator of the acute phase reaction. IL-6 is raised in amniotic fluid of infected preterm labour, stimulates decidual and amniotic PGE_2 production and may be important in the pathophysiology of chorioamnionitis (Romero *et al* 1990; Mitchell *et al* 1991). Countless studies have shown that cytokines are produced in reproductive tissues, some of which provide clear evidence of their role in parturition. Cervical and uterine isthmic biopsies were found to produce far greater quantities of IL-1, TNFα, IL-6 and IL-8 (Winkler *et al* 1998; Sennstrom *et al* 2000; Young *et al* 2002; Osman *et al* 2003; Tornblom *et al* 2005) in labour and postpartum. The same was shown in trophoblast and cervical secretions with raised IL-1β, IL-6, TNF-α, PGE_2 and $PGF_{2\alpha}$ concentrations in labour (Steinborn *et al* 1995).

Figure 1.6 *Proposed fibroblast role in cervical ripening*



Interleukin-8 (or neutrophil activating factor-NAF, neutrophil chemotactic factor-NCF, monocyte derived neutrophil chemotactic factor-MDNCF) is a 99 amino-acid non-glycosylated peptide which is cleaved to release a 72 amino-acid compound. IL-8 is produced by monocytes, macrophages, endothelial cells, lymphocytes, fibroblasts, choriodecidual cells and neutrophils. The main function of IL-8 is to induce chemotaxis, activation and degranulation of neutrophils, releasing MMP-8 and elastase. IL-8 production is up-regulated by IL-1, TNFα, LPS, hypoxia and viral or bacterial infection, and suppresssed by IL-10, dexamethasone and progesterone (Kelly *et al* 1994).

IL-8 is produced in human cervices (Barclay *et al* 1993; Sennstrom *et al* 2000; Young *et al* 2002), is chemotactic for neutrophils, is regulated by IL-1 (El Maradny *et al* 1996) and can produce cervical ripening in rabbits (El Maradny *et al* 1994) and guinea-pigs (Chwalisz *et al* 1994). The changes seen with these animals models are increased water content, neutrophil influx, reduced collagen concentrations, increased activity of collagenase, gelatinase, and elastase (El Maradny *et al* 1996). Not all of these variations have been confirmed in PGE_2 models and there is speculation about a different mechanism of action or a synergism between IL-8 and PGE_2. Critchley et al confirmed the perivascular location of IL-8 in endometrium and this is consistent with a proposed synergistic role with PGE_2 in leukocyte trafficking (Critchley *et al* 1994).

Using lower uterine segment biopsies as a substitute for cervical biopsies, later studies confirmed this exaggerated IL-8 production in association with labour (Osmers *et al* 1995; Winkler *et al* 1998) and this also correlates with raised MMP-8 and MMP-9 levels.

IL-8 produced by cervical fibroblasts has a dual function to induce neutrophil activation and migration and cause degranulation to release collagenase. Other pro-inflammatory cytokines (TNFα and IL-1,) stimulate IL-8 and also have a similar effect on PGE_2 (Barclay *et al* 1993; Sato *et al* 2001). A synergistic action between IL-8 and PGE has been proposed where PGE enhances vasodilation and vascular

permeability for IL-8 augmented neutrophil recruitment. IL-8 is a potent neutrophil chemo-attractant and MMP release (Ledingham *et al* 1999a) from the cervix is known to be associated with IL-8, IL-1 and PGE (El Maradny *et al* 1996). Rabbit cervices respond to local IL-8 application with increased neutrophil numbers, reduced collagen content, increased total GAG and water content and cervical softening (El Maradny *et al* 1994).

In cervical biopsies from labouring women, an increase in IL-8 gene expression and protein release was seen in labour and postpartum (Sakamoto *et al* 2004 ; Tornblom *et al* 2005), accompanied by increased neutrophil numbers, MMP-8 and MMP-9 concentrations (Winkler *et al* 1999b). This supports the chemotactic and proteolytic role of IL-8. Indeed, animal models have produced cervical ripening using locally applied IL-8 or IL-1β (Chwalisz *et al* 1994; El Maradny *et al* 1994; 1996; Belayet *et al* 1999). Whilst MMPs are produced by stromal cells and leukocytes, it seems that cytokines also stimulate smooth muscle cell MMP release (Watari *et al* 1999). The same cells produce IL-8 in response to LPS and may have a specific role in infection associated parturition (Watari *et al* 2003).

Monocyte chemotactic/ chemoattractant protein-1 (MCP-1), alias MCAF/ monocyte chemotactic and activating factor, is a CC chemokine that attracts and activates monocytes but not neutrophils, controls vascular smooth muscle cells, and can modulate T helper cell cytokine production (Baggiolini *et al* 1995). MCP-1 plays a significant role in pathological inflammatory processes (e.g. atherogenesis, SLE, psoriasis, immune response to microorganisms) and there are postulated roles in pregnancy implantation, parturition and ovulation. In addition the presence of MCP-1 has been shown in the human pregnant cervix (Denison *et al* 2000) in labour (Tornblom *et al* 2005).

MCP-1, a 76 amino-acid protein with variable glycosylation, binds to receptor CCR2 which is coupled to a GTP binding protein (Carnevale *et al* 2001). MCP-1 is produced by monocytes, pericytes, fibroblasts, endothelial cells, smooth muscle and synovial cells (Yoshimura *et al* 1990) and is secreted by human endometrium and

placenta (Kelly *et al* 1997). MCP-2 and MCP-3 share 62% and 71% homology with MCP-1 and also specifically attract monocyte, basophils and T-lymphocytes but are less potent (Uguccioni *et al* 1995; Proost *et al* 1996) and had not until recently been identified in the human cervix (Takemura *et al* 2004).

LPS, IL-1, TNF-α, interferon-γ and hypoxia induce MCP-1 synthesis via stimulation of the transcription factor NFκB (Galindo *et al* 2001). MCP-1 is also autoinductive, where migrated monocytes produce MCP-1, perpetuating further chemotaxis (Vestergaard *et al* 1997). MCP-1 induces persistent directional monocyte migration after only brief stimulation (15-30 mins) through interaction with CCR2 receptor (Kito *et al* 2002). Glucocorticoids, IL-10 and progesterone are inhibitory whereas 17β-oestradiol inhibits MCP-1 induced chemotaxis *in vitro* (Yamada *et al* 1996; Kelly *et al* 1997).

Functions of MCP-1 include monocyte chemotaxis, angiogenesis, tumour suppression, regulation of cytokine expression and immunomodulation (Conti *et al* 2001). In addition, MCP-1 triggers firm adhesion of monocytes to vascular endothelium (Gerszten *et al* 1999) and induces transforming growth factor-β (TGF-β) and procollagen gene expression in rat lung fibroblasts (Gharaee-Kermani *et al* 1996).

MCP-1 upregulates both MMP-1 and TIMP-1 mRNA and protein synthesis via an autocrine IL-1α loop in human dermal fibroblasts (Yamamoto *et al* 2000). This may provide a mechanism of action within the human cervix. MCP-1 has also been shown to activate monocytes, stimulating arachidonic acid release and fibroblast IL-1 production in human skin (Yamamoto *et al* 2000). The resident fibroblast is able to initiate or amplify inflammatory responses by chemokines and cytokine release. This is a potential direct mechanism of action of MCP-1 on cervical fibroblasts as it could attract and activate monocytes, stimulate cytokine and matrix metalloproteinase release and contribute to cervical remodelling.

1.4.7 Nitric Oxide

Nitric oxide (NO) is a freely diffusible inflammatory mediator produced from L-arginine by several NO synthases. Endothelial and neuronal NOS (e/nNOS) are constitutive and Ca/calmodulin dependent whereas the inducible NOS (iNOS) is Ca-indpendent. Inflammatory mediators regulate NO release via cGMP and NFκB. NO behaves differently to many other inflammatory mediators as its diffusible nature, and short half life, allows direct effects determined by chemical reactivity and site of synthesis. NO effects are regulation of vascular tone, platelet aggregation, vascular permeability, apoptosis and smooth muscle relaxation. NO also stimulates IL-8, MMPs, LHRH, CRH and has a variable effect on COX-2.

The mechanism of action of NO involves intracellular GMP augmentation and effects include smooth muscle relaxation (by myosin light chain dephosporylation), increased vascular permeability and immunomodulation via IL-8 and MMPs. iNOS synthesized NO acts via both cGMP and other immune and inflammatory pathways. The myometrial effects are thought to be protective during gestation in maintaining uterine quiescence.

NO also induces COX-2 and therefore PGE_2. There are several similarities between PGE_2 and NO regulation in that both respond to cytokine induction and synthesis is controlled by inducible enzymes present in inflammatory cells. Synthesized by macrophages, myometrium, cervix, fetal membranes and other cell types such as cardiac muscle, nitric oxide synthesis is stimulated by proinflammatory cytokines IL-1α, TNFα, IFNγ and LPS, oestrogen and inhibited by dexamethasone. However PGE_2 can have either influence, as can progesterone. The relationship between progesterone and NO is particularly relevant as in concert they maintain uterine quiescence and the feto-placental circulation, and with luteolytic progesterone withdrawal in the rat this NO driven uterine inhibition is removed (Buhimschi *et al* 1996; Kublickiene *et al* 1997). However COX inhibitors do not block antiprogestin induced cervical ripening, and perhaps this relates to the overlapping pathways of NO and PGE_2 synthesis. Where preterm labour is associated with infection,

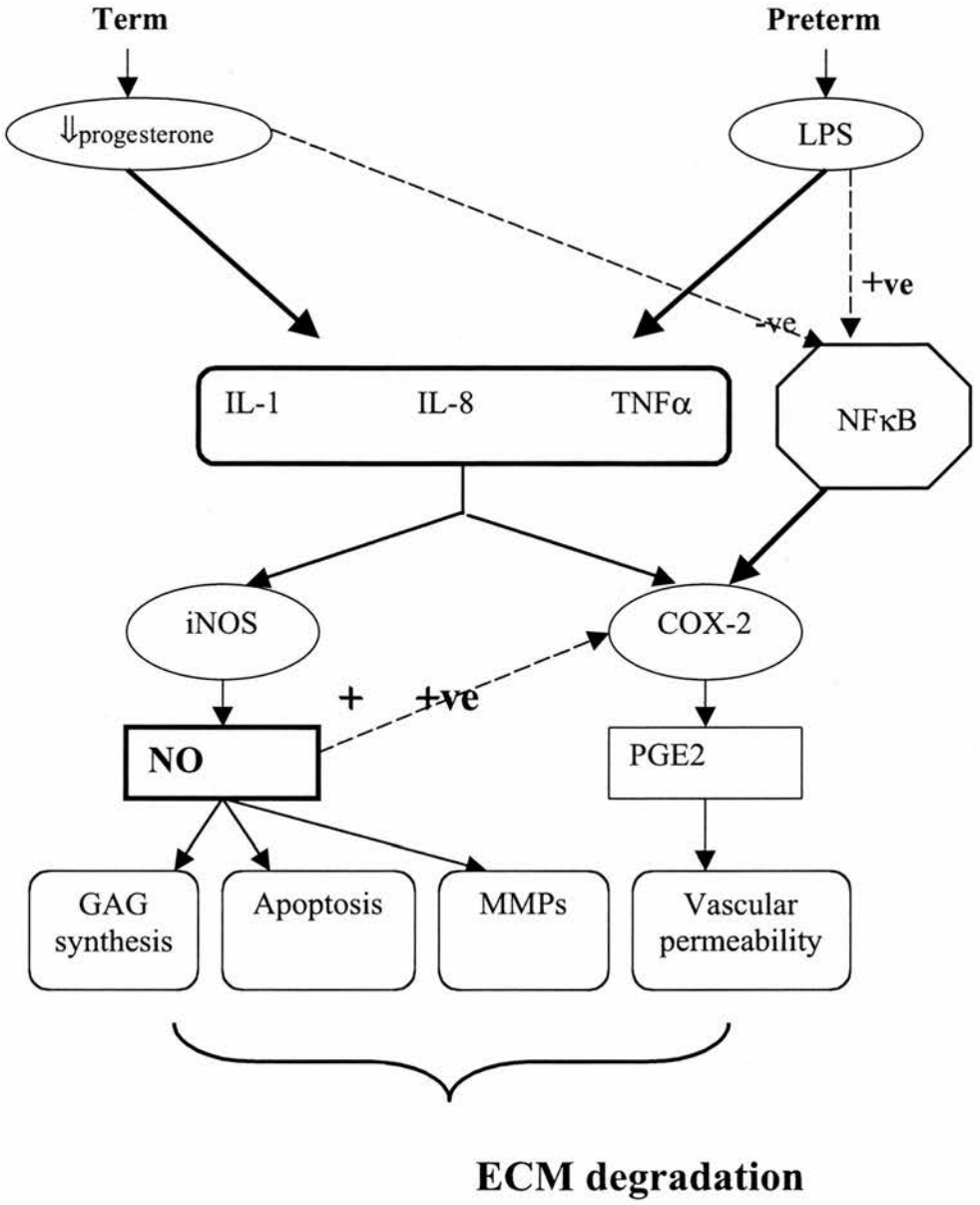
proinflammatory cytokines can directly induce iNOS, COX-2 and PGE_2 without affecting progesterone. And progesterone can also inhibit cervical ripening by suppressing iNOS and COX-2 expression, cytokine release and MMPs.

There is conflicting evidence about the role of NO in labour, but several studies have shown cervical ripening effects in humans and guinea pigs (Chwalisz *et al* 1997; Thomson *et al* 1998; Facchinetti *et al* 2000). However these findings were in early human pregnancy and mid-gestation in the guinea pig study, so similar effects would have to be shown at term. It is possible that there are different mechanisms involved in the initiation of term and preterm labour. See Figure 1.7

In rats, uterine and placental iNOS and NO production is seen to increase in pregnancy and then down-regulate before term whereas cervical NO production is the opposite (Buhimschi *et al* 1996; Chwalisz *et al* 1998). Buhimschi also showed that onapristone (an antiprogesterone) suppressed uterine but increased cervical NO production, whereas progesterone had the reverse effect. iNOS, eNOS and nNOS are present in the rat cervix but only iNOS and eNOS in the uterus.

The same has not yet been clarified in humans as all three isoforms are present in the myometrium yet myometrial and placental NOS activity was not reduced in labour in comparison to those delivered electively (Thomson *et al* 1997a; Bao *et al* 2002). Another study found myocyte iNOS expression increased in pregnancy prior to a marked peripartum reduction (Bansal *et al* 1997). Human cervical NOS (iNOS, eNOS and nNOS) expression increases thoughout gestation pre-labour with a particular late rise in iNOS (Ledingham *et al* 2000). Localisation studies found iNOS and eNOS in the vascular endothelium, nNOS in superficial epithelium in early pregnancy and at term in stromal leukocytes (iNOS and nNOS) (Ledingham *et al* 2000). The mechanism of action is still in doubt and although does not appear to be via MMP-2/-9 could be via other MMPs, a role in apoptosis or interaction with COX-2/ prostaglandin pathways. NO metabolites are increased in human cervical fluid at term, after cervical ripening and with labour (Vaisanen-Tommiska *et al* 2003). NOS activity is seen in first trimester cervical endothelium, smooth muscle

Figure 1.7 *Mediators of term and preterm cervical ripening*



(adapted from Chwalisz and Garfield 1998)

and in the connective tissue and NO donors inhibited contractility in-vitro (Ekerhovd *et al* 1998). Cervical NO production is low in pregnancy, then upregulates toward term, and NO donors are effective cervical ripeners in animal and first trimester human studies (Chwalisz *et al* 1997; Thomson *et al* 1997b; Facchinetti *et al* 2000).

One suggestion is that the source of increased cervical NO (seen at term and with labour) is likely to be leukocytes, a plentiful source of NO. An ingress of leukocytes with cervical ripening has been established and iNOS expression is upregulated by cytokines linked with cervical ripening. NO does appear to cause clinical cervical softening in human and ECM changes seen by electron microscope in guinea pig cervices, but perhaps in humans NO has an additive role in cervical smooth muscle relaxation (Chwalisz *et al* 1997; Thomson *et al* 1997b; Ekerhovd *et al* 1998). This would then act synergistically with the ECM degradation produced by PGs.

It must also be recognised that *in vitro* human cervical fibroblasts do produce iNOS in response to IL-1α, with a resultant increase in MMP-1 (Yoshida *et al* 2001). Also, cervical iNOS mRNA augmentation occurs with advancing gestation and labour (Ledingham *et al* 2000). Cultured human cervical fibroblast and explant secretion of MMP-2 and MMP-9 respectively, were not altered by NO donor treatment (Ledingham *et al* 1999a), although samples were from non-pregnant or first trimester cervices. The lack of MMP-9 in cultured fibroblasts probably reflects the likely bone marrow-derived cell of origin of this particular MMP. So the mechanism of NO induced cervical ripening is still under investigation, whether there be other factors to consider or there are gestation dependent changes not revealed by this work.

Importantly NO also appears to have a significant role in maintaining the feto-placental circulation and so any therapeutic candidates would need to be specific to a site of action. The benefit of NO donors for cervical ripening would be the absence of uterine activity as seen with PGE_2.

Systemic NO inhibits uterine contractility whereas local application appears to cause cervical ripening. *In vitro* studies also reveal NO's capability to inhibit human cervical contractile activity (Ekerhovd *et al* 1998) in addition to the biochemical changes seen. Pharmacokinetic studies will be required to assess the effects at different tissue concentrations. There may yet be a role for NO donors or NOS inhibitors in the management of cervical ripening or prevention of preterm labour, but further work is required on bioavailability studies and dose-finding.

1.4.8 Stretch

The presenting part of the fetus subjects the uterine cervix to cyclical mechanical stretch in the later stages of pregnancy in the form of Braxton-Hicks contractions.
One of the effects of stretch is that it upregulates MMP-1 as shown in cultured human cervical fibroblasts (Yoshida *et al* 2002). Takemura et al propose a direct effect of stretch on chemotaxis of neutrophils and monocytes. Their model exposed cultured cervical fibroblasts to cyclical stretch resulting in an increased IL-8 and MCP-3 expression and secretion, a finding that was not replicated in skin fibroblasts, suggesting this is a specific response in cervical fibroblasts (Takemura *et al* 2004). Although this does not directly prove an effect on leukocyte infiltration, both IL-8 and MCP-3 are known chemoattractants and this lends credence to this hypothesis.

1.5 DECIDUALISATION

1.5.1 Endometrial Decidualisation

Decidualisation of the endometrium is a well described phenomenon, essential in preparation for implantation and pregnancy. With persistently elevated progesterone levels after ovulation, the superficial layer of the endometrium undergoes extensive tissue remodelling. The epithelium becomes secretory and the stromal compartment undergoes decidualisation during the late luteal phase. These events are regulated by the action of progesterone on oestradiol-primed, fibroblast-like precursor stromal cells and is coordinated through progesterone-dependent genes.

Decidualisation is initiated in the vicinity of blood vessels before spreading throughout the endometrial stroma. This whole process prepares the uterus for trophoblast invasion or, in the absence of pregnancy, menstruation. And so disturbance of this mechanism may result in menstrual disorders or subfertility.

Decidual stromal cells (DSC) are differentiated endometrial stromal cells and form the main elements of decidual stroma, together with bone-marrow-derived uterine natural killer (NK) cells. There is controversy about the origin of DSC. Some rodent-based evidence suggests that DSC may originate from bone marrow-derived stem cells (Kearns *et al* 1982; Lysiak *et al* 1992), but the obligate presence of the nuclear progesterone receptor in precursor stromal cells would seem to refute this, as this is not a feature of haemopoietic-derived cells.

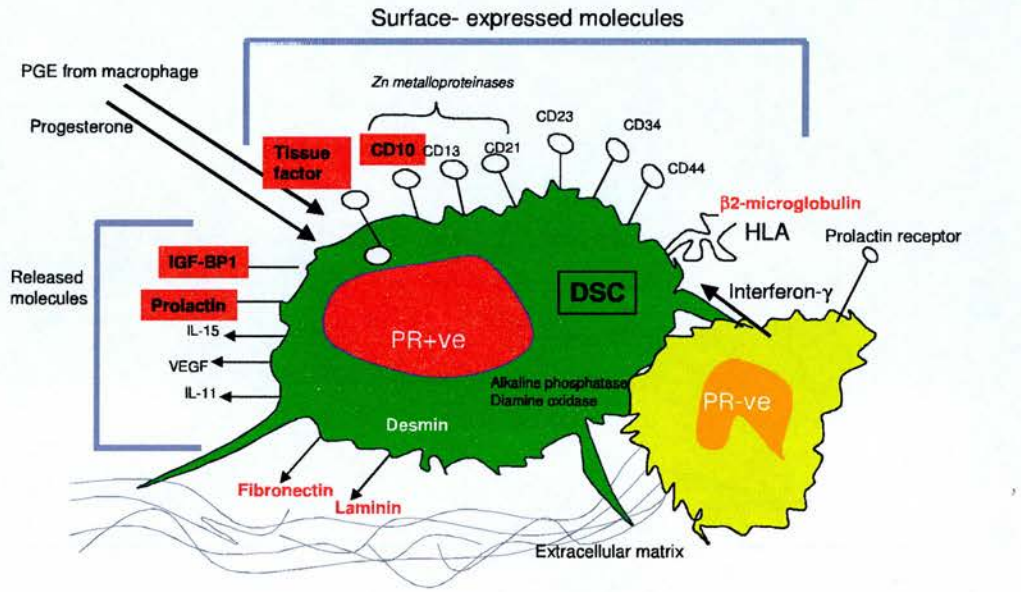
The decidual reaction, which occurs *in vivo* under the influence of prolonged progesterone exposure, occurs more readily *in-vitro* with endometrial stromal cells sensitised to progesterone by elevated cAMP levels (Brosens *et al* 1996; Brar *et al* 1997; Brosens *et al* 1999). *In vivo*, decidualisation coincides with local release of PGE_2 and relaxin, and prolactin (PRL) is first detected in the endometrium at day 10 post-ovulation. This is an environment with sustained high progesterone levels. This process may be seen much earlier *in vitro* where typical patterns of PRL and

IGFBP-1 production is seen from 48 hours (Brosens *et al* 1999; Gellersen *et al* 2003). Many *in vitro* studies have identified the intracellular second messenger cAMP as a putative key factor in the decidualisation process (Tang *et al* 1993; Lane *et al* 1994; Richards *et al* 1995; Telgmann *et al* 1997; Brosens *et al* 1999) and this is further supported by the fact that the phosphodiesterase inhibitor, relaxin, is also an initiator of decidualisation. Intracellular second messengers function by transmission and amplification of cell signals. Cyclic AMP alters gene transcription by activation of the PKA pathway (Telgmann *et al* 1997). Activators of adenyl cyclase (e.g. PGE_2 via EP2 or EP4 receptors) increase ATP conversion to cAMP, and PDE inhibitors (e.g. rolipram, relaxin) prevent cAMP breakdown, thereby maintaining high cAMP levels (Bartsch *et al* 2001).

The decidual phenotype is recognised by a rounded morphology, with enlarged endoplasmic reticulum, abundant glycogen stores and cell projections (Lane *et al* 1994). Decidual stromal cells appear to acquire myofibroblastic characteristics (Oliver *et al* 1999). Characteristic gene expression and protein production is also evident (Tang *et al* 1993; Richards *et al* 1995; Telgmann *et al* 1998). Figure 1.8.

Prolactin (PRL) and Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) are both expressed by decidual stromal cells and are reliable markers of decidualisation (Telgmann *et al* 1998). Prolactin is vital for lactation in the human, and is produced by the anterior pituitary, reproductive tissues and various leukocytes. There may be a local immuno-regulatory role for prolactin in reproductive tissues and steroid interaction is clearly important. Decidual PRL is known to increase to a maximum in amniotic fluid at week 20 of gestation, before a gradual decline to term, but the role of PRL in decidua remains uncertain (Clements *et al* 1977). IGFBP-1 levels rise in both serum and amniotic fluid during pregnancy to a peak in the mid trimester. IGFBP-1 is a good marker of decidualisation and is thought to influence trophoblast invasion by modulating IGF bioavailability (Gibson *et al* 2001).

Figure 1.8 *Decidualisation markers*



Decidualisation encompasses morphological and biochemical alterations. At a molecular level there are many changes in cell function resulting in altered steroid metabolism, cytokine, growth factor and enzyme expression and ECM remodelling. Extracellular matrix reorganisation at the time of decidualisation is vital for trophoblastic invasion. Fibronectin, desmin, laminin and β2-microglobulin are induced in human endometrial decidualisation (Aplin *et al* 1988; Loke *et al* 1989; Komatsu *et al* 1998) and MMP variation occurs in response to progesterone modulation (Lockwood *et al* 1998). Matrix protein deposition is essential for implantation and there is evidence of increased expression of desmin, laminin and fibronectin in both human and rodent models (Glasser *et al* 1987; Can *et al* 1995; Korhonen *et al* 1997; Oliveira *et al* 2000).

In addition to endometrial decidualisation various ectopic sites of decidual foci have been reported including the cervix and ovary (Massi *et al* 1995). Indeed extrauterine decidual-like cells have been identified in the human cervix, uterine tube and ovary during pregnancy (Rutanen *et al* 1991). These were identified morphologically and were stain-positive for IGFBP-1 antibody by immunohistochemistry. However the potential for decidual transformation of stromal cells outwith the endometrium has not been further investigated.

The cervical stromal fibroblast cell is possibly a key regulator in the process of cervical connective tissue remodelling throughout pregnancy and labour. Very little is known about how this cell population may change in pregnancy in response to the same sex steroids that initiate decidualisation in the endometrium. Cervical ripening has often been compared to an inflammatory reaction with increased vascularity and enzymatic collagen degradation (Liggins 1981; Sennstrom *et al* 2000) through matrix metalloproteinases (Yoshida *et al* 2002), and more recently associated with an ingress of leukocytes (Junqueira *et al* 1980; Denison *et al* 2000). The stromal fibroblast is the most likely source of collagen and matrix protein production in the cervix, and as a source of chemokines, prostaglandins, and other inflammatory mediators, so these cells may have a key role in cervical remodelling (Sato *et al* 1991). If these stromal cells also differentiate to a decidual phenotype, a new

appraisal of their role in pregnancy and in cervical function throughout gestation is appropriate.

1.5.2 Progesterone Role in Decidualisation

The decidual reaction, for which progesterone is critical, occurs *in vivo* under the influence of prolonged progesterone exposure. *In-vitro* this process occurs more readily in endometrial stromal cells sensitised to progesterone by elevated cAMP levels (Brosens *et al* 1996; Brar *et al* 1997; Brosens *et al* 1999).

Progesterone receptors are members of the superfamily of ligand-inducible transcription factors, and have been identified in human cervices (Gorodeski *et al* 1987; Stjernholm *et al* 1999; Remoue *et al* 2003). There are two isoforms, PR-A and PR-B with differing N terminals (Kalkhoven *et al* 1996). Transcriptional activities are dependent on the target cell. In general PR-A is less active and inhibits PR-B transcription activity. PR-A is dominant in decidua and in cultured endometrial stromal cells, where there is an initial increase with cAMP/MPA stimulus, and a later prolonged down-regulation with sustained exposure (Brosens *et al* 1999). Stjernholm et al demonstrated a reduction in cervical PR concentration in advanced pregnancy and postpartum but the isoforms were not distinguished (Stjernholm *et al* 1997; Stjernholm *et al* 1999).

Decidualisation requires the presence of progesterone receptors and as such is restricted to reproductive tissues. It is likely that human cervical stromal cells express the progesterone receptor (PR) as shown in lower uterine segment stromal cells (Perrot-Applanat *et al* 1994; Winkler *et al* 2002), and as such may play a role in the cervical changes seen during pregnancy, such as the increased vascularisation. The elusive "progesterone withdrawal" may be an effect of alteration in PR status or function. There is little evidence of specific cellular localisation of PR receptors (A and B) in the human uterine cervix, and in particular in pregnancy, but this is due to difficulty in obtaining such specimens. Previous immunohistochemical studies have

identified the presence of PR within fibroblasts in the cervical stromal compartment in pregnancy, using the lower uterine segment as a proxy (Winkler *et al* 2002).

Progesterone results in a reduction in MMP-1 and MMP-3 (lytic enzymes) and thus an increase in matrix synthesis with decidualisation (Lockwood *et al* 1998). Progesterone withdrawal also results in up-regulation of these key proteolytic enzymes *in vitro* and would therefore provide the ideal mechanism to induce menstruation, in the absence of pregnancy.

Progesterone levels are thought to restrict excessive neutrophil entry and this has been well described in a sheep model (Staples *et al* 1983). Progesterone antagonism increases PGE_2, chemotactic agents, leukocyte numbers and vascular permeability, which in combination promote significant controlled leukocyte infiltration. VEGF may be partly responsible for the vascular permeability changes in the pregnant cervix as in the endometrium, as expression of VEGF and its receptor is up-regulated in human decidual cells (Sugino *et al* 2002).

It is known that PGE_2 levels increase in pregnancy, but it must also be remembered that PGE_2 catabolism by PGDH is also progesterone dependent (Cheng *et al* 1993). PGE_2 and relaxin have both been used to elicit cervical ripening and both are associated with endometrial decidualisation. Alteration of the PGE, cAMP and progesterone pathways have been shown at different stages in pregnancy in uterine tissues. It seems likely that interaction between these pathways provides a physiological decidual stimulus via cAMP up-regulation in association with a progesterone rich environment. Progesterone withdrawal then results in endometrial menstruation and a similar mechanism in the cervix may contribute to the cervical remodelling associated with late pregnancy and labour.

The stages that comprise early implantation are apposition and adhesion of the blastocyst to the uterine lumen, penetration of the epithelium, enhanced decidualisation of the stromal cells and trophoblastic invasion into the stromal vasculature. The fact remains that although many cellular, biochemical and

molecular associations with endometrial decidualisation have been confirmed, the purpose and function of this decidual phenotype is still largely unknown. It appears that MMP and matrix protein modulation occurs in concert with tissue and vascular remodelling and angiogenesis effecting appropriate trophoblast invasion and fetal cell migration. Insufficient invasion results in miscarriage or pre-eclampsia and excessive invasion results in the highly dangerous condition of placenta percreta. The specific functional role of IGFBP-1 or PRL remains unclear and the control mechanisms are unknown. Prolactin, produced by human DSC could activate receptors on uterine large granulated lymphocytes potentially influencing their role in post-implantation uterine function (Gubbay *et al* 2002) whereas IGFBP-1 is thought to influence trophoblast invasion by modulating IGF bioavailability (Gibson *et al* 2001).

All the above considerations lead to the question: does cervical "decidualisation" exist and is it a pre-requisite for successful pregnancy and appropriate gestation-dependent cervical remodelling? Parallels may be drawn, but there are also contrary functions in these two tissues. In particular the cervical stroma is not required to accommodate trophoblast invasion or the extent of vascular remodelling seen in the cervix. Could the decidual response be a reproductive stromal cell state that becomes tissue specific dependent on other factors such as receptor status and transcription factors? The cervical "decidual" response could be distinct. Here cytokine, growth factor, matrix protein production and MMP modulation may render the cervical stroma sensitive to other triggers such as cyclical stretch and functional progesterone withdrawal at term.

1.6 SUMMARY

Cervical remodelling associated with pregnancy and labour is a complex physiological process and there are many contributory factors. Current clinical interventions are limited by gaps in our knowledge of the process and by difficulty in and limitations of administering cervical ripening agents. A series of clinical studies with the needleless injector were planned but did not progress beyond the first proof of principle study (see Chapter 3). Further study of the role of the cervical fibroblast in pregnancy is the major focus within this thesis as a result of this change in direction. Human cervical tissue is notoriously difficult to obtain, but further studies must address the detail of cervical function with respect to the different cell types present in the cervix and their interactions. The aims of this work are to explore the hypothesis that the human cervix undergoes a preparatory phase in gestation akin to endometrial decidualisation, that the key cell involved is the cervical fibroblast and that the fibroblast may facilitate leukocyte recruitment by an MMP-14 induced proMMP-2 activation pathway.

1.6.1 Hypothesis

The proposal is that the cervical fibroblast/ stromal cell has a central role in cervical remodelling witnessed throughout pregnancy and in labour. Central to cervical ripening is the rearrangement of the stromal ECM and therefore stromal tissue distribution of active drug is essential. Reliable and efficient drug administration could be improved by direct administration to this target area. A novel method of needleless injection to the cervix may provide this if stromal penetration is achieved. Evidence supports the fact that the cervical fibroblast has the capability to respond to cytokines and steroids, the capacity to produce collagen, cytokines, chemokines, MMPs and thus play a key role in tissue remodelling. Cervical fibroblasts do appear to behave differently in pregnancy and are progesterone responsive. What is not yet clear is whether there is phenotypic variation in the cervical fibroblast of pregnancy,

as is seen in endometrial stromal cells. If this were so then this phenotype must assessed in order to appreciate subsequent responses and functions in pregnancy and labour. In addition MMP-14, as an activator of MMP-2 and MMP-8, may have a specific role in cervical remodelling when one considers the potentiation of leukocyte recruitment.

1.6.2 Aims of this thesis

1. To assess a needleless injection method for drug delivery to the cervix.
2. Assess the response of the cervical fibroblast to decidual stimuli.
3. Characterise any cervical fibroblast decidualisation-like response.
4. Investigate MMP-14 expression in the human cervix.

CHAPTER 2

GENERAL MATERIALS AND METHODS

MATERIALS

TISSUE COLLECTION

RPMI 1640 culture medium	Sigma-Aldrich Co Ltd, Poole, UK
Tissue culture flasks	Costar Ltd, High Wycombe, UK
Neutral Buffered Formalin (NBF)	See appendix II
70% Ethanol	

TISSUE CULTURE MATERIALS

RPMI 1640 culture medium	Sigma, Poole, UK/ PAA Laboratories
Fetal calf serum (FCS)	PAA Laboratories, Teddington, UK
Phosphate buffered saline (PBS)	Sigma, Poole, UK
Penicillin/ Streptomycin	Sigma, Poole, UK
Gentamicin	Sigma, Poole, UK
L-glutamine	Sigma, Poole, UK
Complete medium	See appendix II
2% FCS medium	See appendix II
Prostaglandin E_2	Control Therapeutics, East Kilbride, UK
Medroxy progesterone acetate	Sigma, Poole, UK
Oestradiol	Sigma, Poole, UK
RU486/ Mifepristone	From Dr D Philibert, Roussel-UCLAF
Rolipram	Affiniti Research Prods. Ltd, Exeter, UK
Lipopolysaccharide	Sigma, Poole, UK
Interleukin-1β	Peprotech Ltd, London, UK
Interleukin-8/ CXCL-8	R&D Systems, Oxford, UK
8-bromo-Cyclic-adenosinemonophosphate	Sigma, Poole, UK
Monocyte chemotactic protein-1	R&D Systems, Oxford, UK
Trypsin	PAA Laboratories, Teddington, UK
Phorbol 12-Myristate 13-Acetate	Sigma, Poole, UK
Freeze mix	See appendix II
Dimethylsulphoxide (DMSO)	Sigma, Poole, UK
Cryovials	Nunc International
Tissue culture flasks	Costar Ltd, High Wycombe, UK
6/12/24 well culture plates	Costar Ltd, High Wycombe, UK

CELL LINES

U937 cells	European Collection of Cell Culture
HS27 cells	European Collection of Cell Culture

RNA EXTRACTION

Tri Reagent	Sigma, Poole, UK

Phase lock gel eppendorfs	Eppendorf AG, Hamberg, Germany
Chloroform	BDH Laboratories Supplies, Poole, UK
Isopropanol	Sigma- Aldrich, Poole, UK
70% ethanol	Hayman Ltd, Essex, UK
RNA storage buffer	Ambion

REVERSE TRANSCRIPTION

0.6ml PCR tubes	
Reverse Transcription kit	Applied Biosystems, Warrington, UK
TE buffer	See appendix II
Mineral oil	Sigma, Poole, UK

POLYMERASE CHAIN REACTION

Taqman Polymerase Chain Reaction kit	Stratagene, Amsterdam, Netherlands
0.6ml PCR tubes	
96 well optical plates	Applied Biosystems, Warrington, UK
Optical cap strips	Applied Biosystems, Warrington, UK
18S primer/probe set	Applied Biosystems, Warrington, UK
All other primer/probe sets	Biosource, Nivelles, Belgium

ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

Plates (96 well)	Nunc Maxi-Sorp, Gibco, Paisley, UK
Wash buffer	See appendix II
Blocking solution	See appendix II
Dry coat	See appendix II
Donkey anti-rabbit serum	Scottish Antibody Production Unit, Law Hospital, UK
ELISA assay buffer	See appendix II
Streptavidin peroxidase	Roche, Lewes, UK
Sulphuric acid (H_2SO_4)	BDH
Substrate	See appendix II

Interleukin-8 (IL-8) ELISA

IL-8 capture antibody (mouse monoclonal)	R&D Systems Ltd, Oxford, UK
IL-8 recombinant standard	R&D Systems Ltd, Oxford, UK
IL-8 biotin-labelled detection antibody	R&D Systems Ltd, Oxford, UK

IL8 KIT ELISA

Quantikine human IL-8 immunoassay	R&D Systems Ltd, Oxford, UK

MCP-1 ELISA

MCP-1 capture antibody	R&D Systems Ltd, Oxford, UK
MCP-1 standard	R&D Systems Ltd, Oxford, UK
MCP-1 biotin-labelled detection antibody	R&D Systems Ltd, Oxford, UK

IGFBP-1 ELISA

IGFBP-1 capture antibody	R&D Systems Ltd, Oxford, UK
IGFBP-1 recombinant standard	R&D Systems Ltd, Oxford, UK
IGFBP-1 biotin-labelled detection antibody	R&D Systems Ltd, Oxford, UK

PROLACTIN ELISA

Delfia Prolactin kit	Perkin Elmer Applied Biosysytems

FACS

CD10 phycoerythrin labelled antibody	Beckman Coulter, High Wycombe, UK

NEEDLE-FREE INJECTOR STUDIES

Needle-free injector devices	Weston Medical Ltd, UK
Methylene blue dye	Sigma-Aldrich
Phosphate buffered saline	Sigma-Aldrich

METHODS

2.1 SAMPLE COLLECTION

2.1.1 Uteri

All uteri with cervices (n=6) were obtained intact from healthy non-pregnant women (aged 31-45yrs) undergoing total hysterectomy for benign pathology. All were parous and had had at least one previous vaginal delivery. The Lothian Research Ethics Committee and the Lothian University Hospitals NHS Trust approved the study and use of facilities. Written informed consent was obtained from each subject after consideration of an information leaflet.

Uteri were collected fresh from theatre, transferred to the laboratory and returned to theatre within 60-90 minutes where they were fixed in formalin and processed normally through the pathology laboratory.

2.1.2 Cervical Biopsies

Cervical biopsies were obtained from both pregnant and non-pregnant women. For both groups, Lothian Research Ethics Committee and Lothian University Hospital NHS Trust management granted approval. Women were approached in the out-patient setting and given information leaflets to consider before written informed consent was obtained on admission.

Non-pregnant women

Healthy non-pregnant pre-menopausal women aged 32-47 years undergoing total abdominal hysterectomy for benign pathology, were approached. Samples were obtained on day 3-20 although a few cycle stages were unidentifiable due to erratic bleeding. Non-pregnant samples were obtained immediately post-operatively in the Department of Obstetrics and Gynaecology, Royal Infirmary of Edinburgh. Cervical biopsies (n=20) were retrieved from uteri immediately post hysterectomy. A thin (~2mm) section was cut longitudinally through the anterior lip of the uterine cervix and placed in RPMI 1640 medium (Sigma, Poole, Dorset, UK) on ice for transport.

Initial biopsies were processed for cervical stromal cell culture only. Later biopsies were divided and:

1. Processed for cervical stromal cell culture (n=12).

2. Placed in 10% Neutral Buffered Formalin (NBF) at 4°C to fix overnight, then transferred to 70% ethanol for storage until routine paraffin embedding (n=12).

3. Washed with PBS, minced and placed in trireagent for whole tissue RNA extraction (n=5).

Pregnant women

Healthy pregnant women (aged 18-34yrs) at 7-12 weeks gestation as determined by last menstrual period and/or ultrasound scan, attending for surgical termination of pregnancy under general anaesthetic, were approached. All required a negative smear history and were chlamydia negative. Seven of the thirteen had received misoprostol priming as per department protocol, but these samples were only used for cell culture. The brief exposure to misoprostol (1-3hrs) was not likely to have a prolonged effect in view of the length of time required for culture prior to experimental protocol.

Cervical biopsies (n=13) were taken from the anterior lip of the cervix immediately prior to termination, using Shumaker biopsy forceps and placed in RPMI 1640 on ice for transport (Denison *et al* 2000). Shumaker biopsy forceps produce a sample approximately 5mm diameter.

Initial biopsies were processed for cervical stromal cell culture only. Later biopsies were either used for one of the below, or divided if large enough:

1. Processed for cervical stromal cell culture (n=8).

2. Washed with PBS, minced and placed in trireagent for whole tissue RNA extraction (n=6). None in this group had received misoprostol priming.

3. Placed in 10% Neutral Buffered Formalin (NBF) at 4°C to fix overnight, then transferred to 70% ethanol for storage until routine paraffin embedding (n=8). Due to time constraints these samples were never processed for paraffin embedding, hence planned immunohistochemistry studies were not performed.

2.2 TISSUE CULTURE

2.2.1 Cervical stromal cell culture preparation

Both pregnant and non-pregnant cervical biopsies were twice washed in PBS, then dissected into small fragments with scalpel blades and replaced into RPMI complete medium Tissue was then placed in complete culture medium (RPMI 1640 supplemented with 10% FCS, 20µg/ml gentamicin, 100IU/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine), in 25cm^2 tissue culture flasks, at 37°C for 95% air : 5% CO_2 incubation. This resulted in outgrowth of cervical fibroblast-like cells within 7 days and with medium exchange every 3-4 days, the cells grew to confluence. After transfer into 75cm^2, then 162cm^2 flasks the cells had grown to confluence within a maximum of 21 days.

Cervical stromal cells were split 1:5 with standard trypsin:EDTA and were used up to passage 7. Passages varied due to differing sample collection times, culture times per sample, and a need to achieve adequate cell numbers concurrently for the proposed treatment protocols. Passage variation within each study was kept to a minimum. Culture times and passage numbers are consistent with other investigators (Yoshida *et al* 2001; Yoshida *et al* 2002; Takemura *et al* 2004).

Cultured stromal cells were morphologically of fibroblast cell type and responded to monoclonal anti-fibroblast antibody (Oncogene Research Products), which recognises a fibroblast specific surface antigen. Culture conditions used were similar to those for the extensively studied endometrial stromal cells in these laboratories.

Cervical stromal cell storage

Where appropriate cells were frozen in liquid nitrogen using a cryopreservative medium (FCS with 10% dimethylsulfoxide (DMSO). Cervical stromal cells grown to confluence in a large flask, and not for immediate use were washed with PBS, then trypsinised to free them from the plastic surface. A small volume of RPMI was

added and after centrifugation at 1700rpm for 3 minutes a cell pellet was obtained. On ice, the supernatant was then removed and 4-5mls of cryopreservative medium added to re-suspend. Aliquots of 1ml of suspended cells per cryovial were placed at –70°C in a slow freeze apparatus, and after at least 24 hrs the cryovials were transferred to a liquid nitrogen container. On retrieval, cells were recovered in complete medium and grown to confluence within 7-11 days.

2.2.2 U937 monocyte cell line

Undifferentiated U937 cells (ECACC) were used as a model to investigate cytokine and matrix metalloproteinases release. U937 cells are a myeloid-derived "immortalised" cancer cell line. They are pro-monocytic, display many monocytic characteristics and can be induced to macrophage differentiation in vitro. These cells were cultured in complete medium as maintenance and used at a cell density of $1x10^5$ cells/ml for treatment regimes.

2.3 QUANTITATIVE REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION

2.3.1 RNA Extraction

Tri-reagent RNA extraction

1ml Tri-reagent was added to treatment flasks or wells of cultured cells, after the removal of supernatant. RNA was extracted by transferring the Tri-reagent to pre-spun (6000rpm for 2 minutes) phase lock gel-tubes (Eppendorf). They were then placed on ice for 5 minutes to allow lysis. 200µl of chloroform was then added and mixed vigorously, before pre-cooled centrifugation at 14000rpm for 20 minutes at 4°C. The phase lock gel migrates to separate phases of an aqueous/organic extraction; the aqueous layer above the gel contains RNA and the layer trapped below the gel contains DNA and protein. The RNA aqueous phase is transferred to a clean eppendorf 1.5ml tube and mixed by inversion, after the addition of 500µl of 100% isopropanol. A further incubation at 4°C for at least one hour is followed by a second spin at 14000rpm for 15 minutes. This produces an RNA pellet from which the supernatant is removed and 1ml of 70% ethanol to resuspend. This is centrifuged at 14000rpm for 5 minutes at 4°C. The ethanol is removed and the RNA pellet resuspended in 25µl RNA storage buffer (Ambion).

Whole tissue RNA extraction

Cervical biopsies from both pregnant and non-pregnant women were washed twice in PBS and dissected into small fragments with scalpel blades. This was then placed in Tri reagent, homogenised and frozen at -70°C until RNA extraction completed, as above.

Cervical stromal cell culture RNA extraction

At the end of any treatment period, after removal of medium, cultured cells were washed with PBS and then Tri-reagent added to each well/ flask. This was then transferred into phase-lock eppendorfs with chloroform, spun and followed by precipitation with isopropanol. The concentration of total RNA in the samples was

quantitated by UV spectrophotometry (Biotech, WPA, Cambridge, UK) and each sample was then diluted to 100ng/µl. and either reverse transcribed to cDNA or stored at –70°C.

U937 cell RNA extraction

As undifferentiated U937 cells are non-adherent and remain in suspension, cells in medium are collected and spun at 1700rpm for 3 minutes before resuspension in Tri-reagent and RNA extraction as detailed above.

RNA quantification

A 5µl aliquot of each sample was diluted in 2mls diethylpyrocarbonate-treated water (depc H_20) and thoroughly mixed. Quantitation was by UV spectrophotometry and all samples diluted to 100ng/µl as follows. The optical densities measured at 260nm and 280nm determined the concentration and purity of RNA present. A blank was used to zero the machine (2.5µl RSB with 2ml depc H_20) with each set of samples. The 260:280 ratio determined the purity with a value of 1.8 or higher indicating a pure RNA sample. RNA concentrations were calculated by multiplying the 260 OD value by the dilution factor and then a factor of 40 (as an OD value of 1 at 260nm is equivalent to 40µg/ml of mRNA). For example if 5µl RNA is diluted in 2mls depc H_20 (dilution factor 400), then:

RNA concentration (ng/µl) = 260 OD value x 400 x 40

Diluted RNA samples were then stored at –70°C for subsequent use.

2.3.2 Reverse Transcription

Generally, 4µl of template mRNA (at 100ng/µl) was reverse transcribed using 16µl of Taqman reverse transcription reagents (AB Applied Biosystems). The RT mix contained random primers, $MgCl_2$ (5.5 mmol/l), dNTPs (1 mmol/l), random hexamers (2.5µmol/l), RNase inhibitor (0.4 IU/µl) and multiscribe reverse transcriptase (1.25 IU/µl). This 20µl mix was covered with mineral oil to prevent evaporation, and incubated sequentially at 25°C for 20 minutes, 48°C for 60 minutes and 95°C for 5 minutes (Omnigene PCR machine, UK). The resulting single-stranded cDNA was then diluted with x1.5 volumes (30µl) of TE buffer and stored at 4°C for imminent use, or at –20°C for later use.

The precision of the RT reaction was calculated within the department by Elena Faccenda (laboratory research support). An mRNA sample was taken and 8 RT reactions set up in 8 separate tubes. A single PCR run on one primer/ probe set compared them and found precision to be 3.65%.

DNA contamination is a major concern as PCR cannot discriminate between RT synthesized cDNA and genomic DNA, thereby risking false positives. Contamination is be minimised by extraction techniques and primer design but can also be easily detected by "no RT" control PCR as follows. The control mRNA samples from each experiment were assessed for possible genomic DNA contamination by β-actin signal detection without prior reverse transcription. A β-actin signal above 27 cycles indicates no major genomic DNA contamination. 27 cycles has been defined as threshold as this represents 3 standard deviations from a mean of samples (King et al. 2000) without major genomic DNA content. Any samples with a value below 27 indicating contamination were excluded from analysis.

2.3.3 Quantitative Real Time Polymerase Chain reaction

The polymerase chain reaction is a primer-directed in-vitro enzymatic reaction used to amplify a specific DNA fragment. Primers are paired and flank a nucleotide sequence of interest. There are three steps involved:

1. thermal denaturation of the target DNA (heat to 94°C)
2. primer annealing of synthetic oligonucleotide primers
3. extension of the annealed primers by DNA polymerase

This cycle is then repeated and with each repitition the number of product fragments is approximately doubled. Because there is an accumulation of the product fragment, there is a plateau beyond which there will be no amplification, because the enzyme required is of a finite supply. PCR thermocyclers automate the entire procedure. The result is a logarithmic amplification of the target sequence.

Reverse transcriptase-PCR uses messenger RNA (mRNA) rather than DNA, is very accurate and sensitive allowing quantification of very low levels of mRNA. The mRNA is reverse transcribed to produce a copy of the original DNA in single stranded form, called complementary DNA (cDNA). This cDNA is mixed with oligonucleotide primers, probes and enzymes and sequentially heated and cooled to achieve the processes described above. Thermal cycling conditions commence with a 2 minute hold at 50°C, followed by 10 minutes at 95°C. This provides a single stranded template. Thereafter each cycle of 40, heats to 95°C for 15 seconds and holds at 60°C to anneal. A specific labelled oligonuleotide probe will identify its complementary sequence. Real-time quantitative PCR detects gene expression of a particular target at the same time as it is amplified.

The amount of specific DNA at the end of each PCR run does not correlate to the target copies in the original sample, but the cycle number at which a threshold amount of product is seen reflects the original amount mRNA present.

The measurement of PCR product is by detection of released fluorescent reporter dye. Forward and reverse primers recognise the sequence of target DNA whilst a dual-labelled probe recognises a particular sequence located between the annealing sequences of the two primers. The labelling is with two distinct fluorescent dyes. One is the reporter dye and the other a quencher dye (TAMRA). The reporter dye fluorescence is suppressed by the quencher dye in the intact probe, as they are in close proximity. During amplification the taq polymerase cleaves the probe, thus separating the quencher dye from the reporter dye and releasing fluorescence, which can be measured. The fluorescence is only detected if the probe target sequence is cleaved, so non-specific amplification is not recognised (see Figure 2.1).

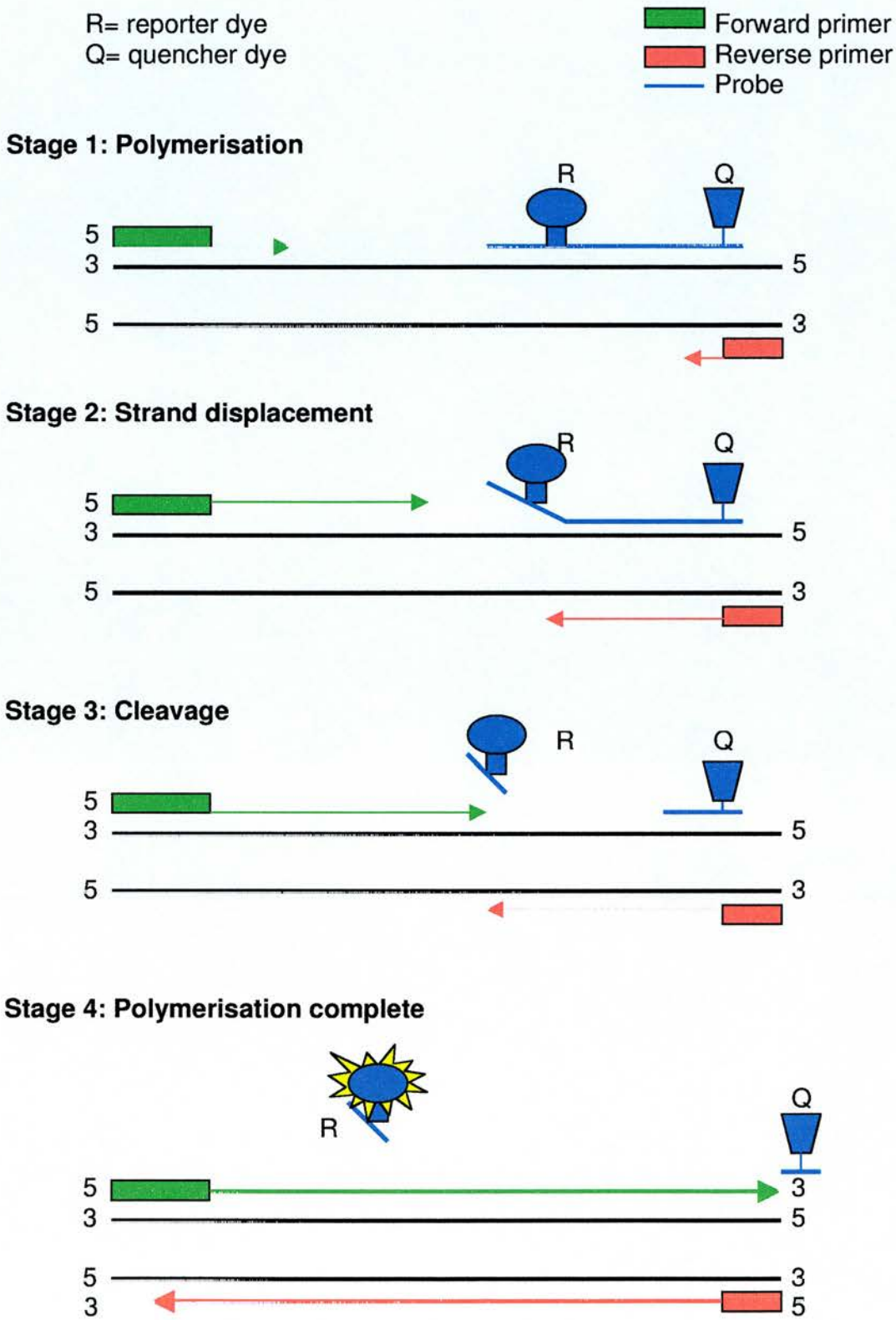
Ribosomal 18-S is used as the internal control. 18-S is constant to the relative amount of cDNA present, and as the endogenous control, is then related to the amount of specific amplicon to normalise variations in cDNA content between samples. A negative control without RNA (depc H_20) was used in each reaction. Ribosomal 18-S and specific amplicons can be measured at the same time as each reporter dye emits a different wavelength. The reporter dye on 18-S is VIC and most specific amplicons use FAM (6-carboxyfluorescein) as the reporter dye.

PCR master mix contained Stratagene Brilliant Quantitative PCR Core Reagent Kit (Amsterdam, Netherlands) (2x PCR buffer, 7.2mM $MgCl_2$, 1.6mM Stratagene dNTP mix, 1.6mM Boehringer dNTP mix, 0.05U/µl sure-start taq polymerase, 0.06% reference dye diluted in depc H_20). All primer/ probe sets were purchased from Biosource (Belgium) and diluted to 250µM (primers) and 50µM (probes) in TE buffer. The mixture was then divided into 45µl (for two replicates) or 67.5µl (triplicate) aliquots per tube. 5µl or 7.5µl (dependent on no. of replicates) of sample cDNA was then added to each tube and mixed. 24µl aliquots were then transferred to a 96 well PCR plate in duplicate or triplicate. A no template negative control (depc H_2O) was also run on each plate. Plates were then sealed with optical caps and the PCR run on ABI Prism 7700 Taqman sequence detector under standard conditions.

PCR data analysis was performed by use of the $2^{-\Delta\Delta Ct}$ formula. Ct refers to the cycle number that the fluorescent signal crosses the threshold. ΔCt is the difference between the specific amplicon (FAM) and the 18-S (VIC) Ct values and enables the amplified signal to be normalised against the total mRNA content. The mean ΔCt between the duplicates on the plate was calculated. ΔΔCt then relates each samples' ΔCt value to its own within experiment control. Therefore, the amount of target mRNA is standardised to the amount of 18-S RNA, and is then compared to its control. This gives a value relating to the fold increase in amount of target mRNA compared to its own control.

Figure 2.1:
Stage 1 shows the probe annealed to the target sequence with the reporter and quencher dyes in close proximity suppressing fluorescence.
Stage 2 shows the forward primer extending along the template and displacing the reporter dye as it reaches the probe.
Stage 3 Taq polymerase (as a 5' endonuclease) cleaves the probe separating the two dyes and increasing the free reporter dye, and therefore fluorescence.
Stage 4 Polymerisation is complete. The fluorescence signal is proportional to the amount of amplicon (PCR product generated), thereby reflecting the initial quantities of the target sequence.

Figure 2.1: *Quantitative real time polymerase chain reaction (Taqman)*



The primers and probes used were designed using the PRIMER express computer program and sequences are listed in table 2.1.

To determine the specificity of each primer/probe set, BLAST nucleotide database searches were performed for each primer and probe (http://www.ncbi.nlm.nih.gov/BLAST/). The returned results constitute a list of 'hits' or DNA sequences found to contain the target sequence. Each 'hit' is accompanied by a 'E' Value (expect rate) which indicates the number of hits expected to be found by chance during the search. The results of the BLAST searches showed that for all sequences of interest, the primer and probe sets used were unlikely to amplify fragments from an inappropriate gene, thereby confirming specificity.

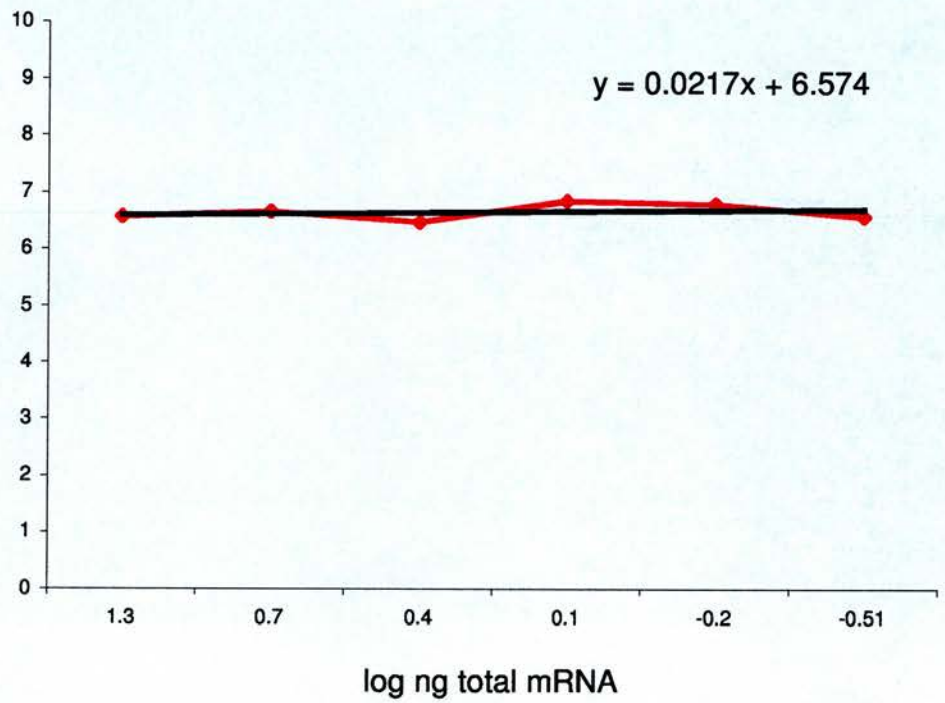
Table 2.1 *Details of the primer/probe sets used in Q RT-PCR*

Amplicon	**Accession no.**	**Forward primer**	**Reverse primer**	**Probe**	**'E'value**
EP2	U19487	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA	CCACCCTGCTGCTGCTTCTCATTGTCT	4×10^{-6}
EP4	D28472	ACG CCG CCT ACT CCT ACA TG	AGA GGA CGG TGG CGA GAA T	ACG CGG GCT TCA GCT CCT TCC T	$*3.8 \times 10^{-7}$
Progesterone receptor (genomic)	M15716	CAGTGGGCGTTCCAAATGA	TGGTGGAATCAACTGTATGTCTTGA	AGCCAAGCCCTAAGCCAGAGATTCACTTT	3×10^{-7}
Prolactin	NM-000948	GCC CCG GAG GCT ATC CTA	TCA GCT CCA TGC CCT CTA GAA	CCA AAG CTG TAG AGA TTC AGG AGC AAA CCA	9×10^{-8}
IGFBP-1	M59316	CACAGGAGACATCAGGAGAAGAAA	ACACTGTCTGCTGTGATAAAATCCAT	TTCCAAATTTTACCTGCCAAACTGCAACAA	9×10^{-8}
VEGF	E15157	TACC TCCACCATGCCAAGTG	TAGCTGCGCT GATAGACATC CA	ACTTCGTGAT GATTCTGCCC TCCTCCTT	1×10^{-6}
Desmin	NM-001927	GGAGAGGAGAGCCGGATCA	GGGCTGGTTTCTCGGAAGTT	TCTCCCCATCCAGACCTACTCTGCCC	1×10^{-5}
Fibronectin	NM-002026	TGTTCGTGCAGCTGTTTACCA	TCTGTGACACAGTGGCCATAGG	CGCAGCCTCACCCCCAGCC	$*3.8 \times 10^{-7}$
β2-microglobulin	NM-004048	GGCACGAGCCGAGATGTC	CCTCCAGGCCAGAAAGAGAGA	CGCTCCGTGGCCTTAGCTGTGC	$*1.5 \times 10^{-6}$
Tissue Factor	NM-001993	CAC CGA CGA GAT TGT GAA GGA	CCC TGC CGG GTA GGA GAA	TGA AGC AGA CGT ACT TGG CAC GGG T	5×10^{-5}
Laminin	NM-000426	CCAGCCTTTCCTACGCCC	AAAGAGCTGGTTCTGATTCTGCA	CCCCAGTTCTGACACATGGTCCTTGTG	4×10^{-6}
CXCL-8	NM-000584	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG	CCTTCCTGATTTCTGCAGCTCTGTGTGAA	3×10^{-7}
PDE4b	L20971	CCTTCAGTAGCACCGGAATCA	CAAACAAACACACAGGCATGTAGTT	AGCCTGCAGCTCCAGCC	
MMP-14	NM-004995	CCC CAG GCG ACT GCT CTA CT	TCG GGA CGA TGG GAA CAG	CCA GCG TTC CCT GCT GGA CAA GG	

E value is for probe alone unless marked by * where E value is for primers and probes combined (each probe E value multiplied by both primer E values).

Primers and probe sets were validated by assessing the linearity of the response in a serial dilution of a standard pool of RNA. The log of total RNA (in ng) was plotted against the mean ΔCt value of three replicates and then a regression line attained. The slope of regression line (y) required was <0.1 in order that the primers and probe be validated. All primer and probes were validated and intra-assay variation calculated as a precision value (based on the mean and relative standard deviation of 6 replicates of identical cDNA in the same run) and expressed as a percentage.
See Figure 2.2.

Figure 2.2 *Linearity plot example*



An example of a linearity plot for the primer/ probe sets used in Q RT-PCR. This figure represents the plot for the fibronectin primer and probes. The y value for this particular plot is 0.02 and is an indication of the linearity.

Table 2.2 *Validation and precision values for the primer and probe sets*

Probe	Linearity (y value)	Precision (%)
β2-microglobulin	0.062	1.3
COX-1	0.053	1.6
COX-2	0.083	2.0
CXCL-8	0.031	4.0
Desmin	0.08	1.3
EP2	0.045	1.5
EP4	0.019	1.0
Fibronectin	0.022	2.4
IGFBP-1	0.099	6.4
Laminin	-0.094	2.8
MCP-1 (CCL2)	0.093	11.4
PDE	-0.001	4.2
PGDH	-0.11	1.4
Progesterone-receptor	0.004	0.8
Prolactin	0.062	2.4
Tissue Factor	-0.17	9.7
vEGF	0.081	1.7

2.4 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

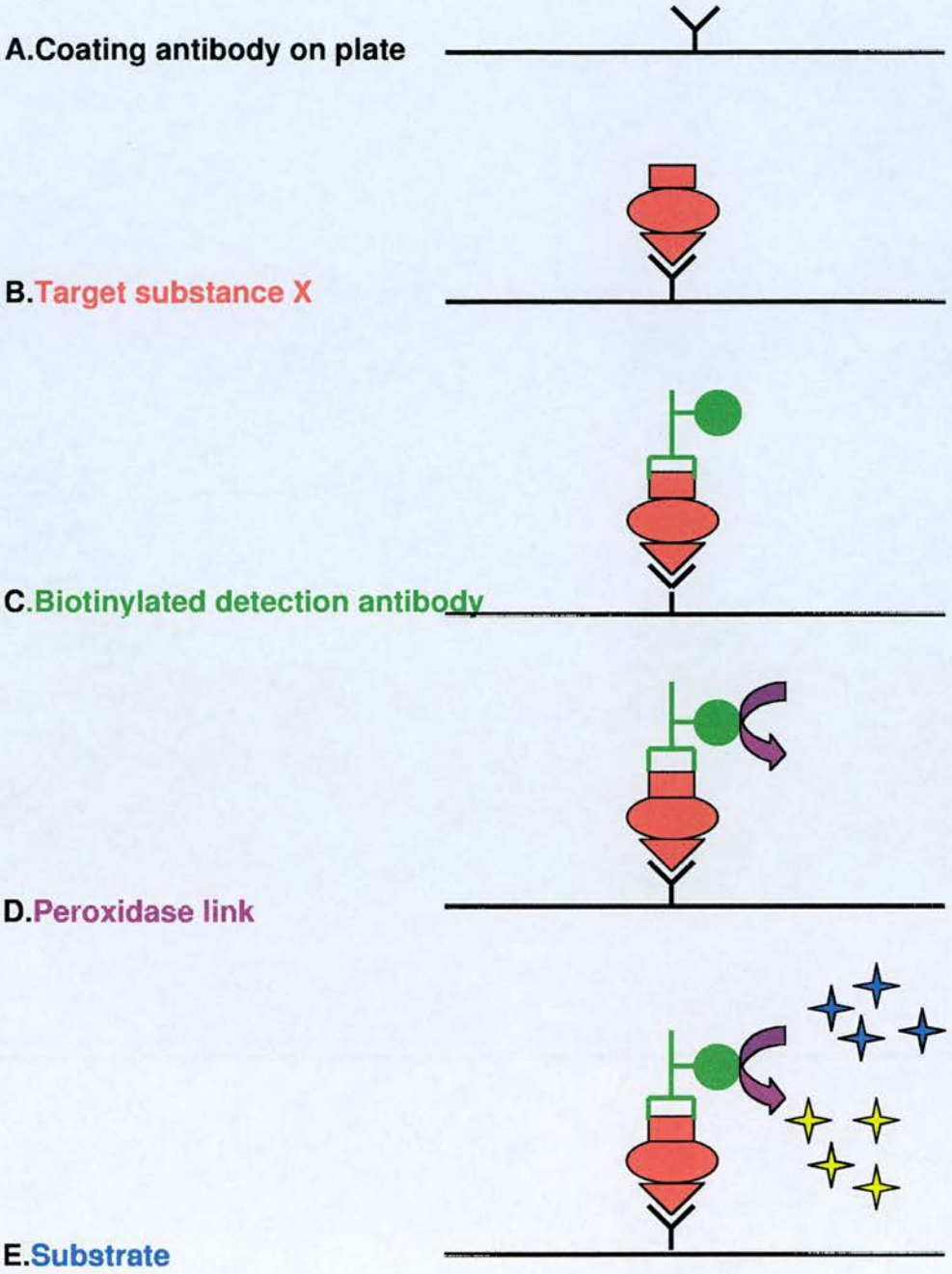
2.4.1 ELISA is a technique whereby the concentration of a protein in solution can be calculated by comparison with a standard curve created from prepared solutions of known concentrations. Two main methods of ELISA are commonly used; the Two Site ELISA and the Competition ELISA. Both types run on Maxisorp 96-well plates. Method files for each ELISA were constructed in Assay Zap with the range of standards used, producing a standard curve against which any samples can be measured.

1. **Two Site ELISA** (eg. IL-8)

This method involves using paired antibodies. A capture antibody coats the plates and biotinylated detection antibody is used to detect the bound substance, which is then quantified by adding streptavidin peroxidase. The peroxidase is then measured by a colour reaction.

96 well plates are initially coated with a coating antibody specific to the protein of interest. Blocking medium minimises non-specific binding and reduces background recordings. The samples, standards, quality controls and non-specific binding wells are then incubated. The target analyte of measurement is recognised and bound at a specific site by the coating antibody. Excess media is then discarded and washed away. The detection antibody recognises the target analyte but is bound by a different specific site. The detection antibody is also biotin-labelled, which is bound by streptavidin peroxidase, when added. Another wash removes any unbound streptavidin peroxidase conjugate. Substrate added then detects the peroxidase, which converts the tetra methyl benzidine into a coloured product (see figure 2.3). The reaction is stopped by the addition of 2N sulphuric acid, before the colour measured. The end result is that your target analyte is sandwiched between two specific antibodies, one of which is biotinylated, binding peroxidase which is detected by the substrate and quantifiable by EIA 450nm plate reader. These values are then compared against the standard curve created to calculate the amount of target substance present in the solution measured.

Figure 2.3 *Two site sandwich ELISA*



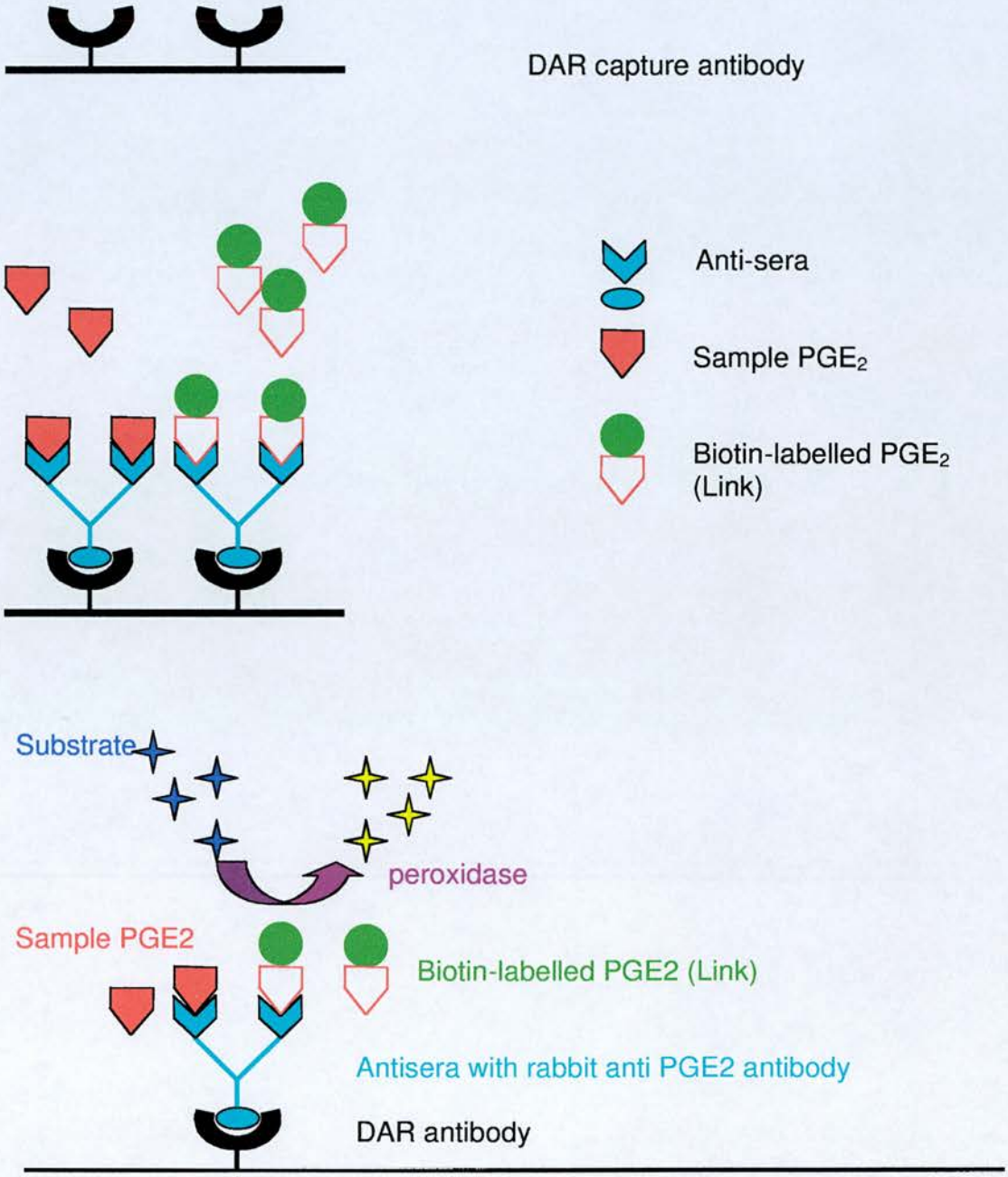
A.Plate coated with capture antibody
B.Target substance bound to capture antibody
C.Biotin labelled detection antibody bound to target substance
D.Streptavidin peroxidase links to biotin labelled detection antibody
E.Peroxidase produces measurable colour on addition of substrate.

2. **Competition ELISA** (eg. PGE_2)

96 well plates are coated with the purified donkey anti-rabbit antibody (Sapu, Scotland, UK) and then blocked. This removes non-specific binding. A PGE_2 anti-sera raised in rabbit is added to the wells which will bind to PGE_2. The solution containing unknown amounts of the analyte is added with anti-sera and link (biotinylated PGE_2). The anti-sera binds with both the recombinant protein and the analyte. The result is competition, between the link-bound PGE_2 and the sample PGE_2, for the anti-sera. Therefore if there are high concentrations of the analyte, more of the link is displaced and less colour displayed (see figure 2.4). Low concentrations of analyte produce high levels of link bound to anti-sera, and a high optical density reading. A secondary antibody binds to a separate site on the anti-sera, which is then identified by the detecting system, to give a colour reaction. High levels of sample PGE_2 increase competition displacement of the biotin labelled link, giving a lower concentration of coloured measurable product.

A standard curve and quality controls are generated to calculate sample concentrations.

Figure 2.4 *Stages and principles of a Competition ELISA (PGE2)*



Competition displacement ELISA. Higher levels of sample PGE_2 result in increased displacement of the biotin labelled link and so a lower concentration of coloured measureable product.

2.4.2 Interleukin-8 ELISA

IL-8 release was assayed by ELISA with matched pairs of capture and biotin-labelled detection antibodies for IL-8. Plates were coated with capture antibody (2μg/ml) at 100μl/well overnight at 4°C. After a single wash, blocking solution was added at 400μl/well for 30 minutes at room temperature. Plates were either washed once and used immediately or, after removal of the blocking solution, air dried and stored with a dessicant at –20°C.

100μl/well of samples and standards were added, standards ranging from 2000-3.9 pg/ml, and samples in duplicate. The plates were incubated overnight at 4°C or at room temperature for 3 hours on an orbital shaker, washed again, and then detection antibody added (50ng/ml; 100μl/well). A further incubation period of 60-90 minutes at room temperature with shaking was followed by a further wash and addition of streptavidin peroxidase (100μl/well). The final wash was followed by the addition of substrate (200μl/well) before quenching with 50μl/well 2N H_2SO_4.
Within 30 minutes of quenching, absorption was read at 450nm. Values are then compared against the standard curve produced, to calculate the amount of IL-8 in each sample. Intra and inter-assay precisions were 9.1% and 22% R.S.D. respectively with a detection limit of 15pg/ml (Denison *et al* 1997).

R & D Quantikine Human IL-8 Immunoasssay

This kit employs the sandwich technique using a specific IL-8 monoclonal antibody pre-coated on the plate. IL-8 in samples and standards is then sandwiched between the immobilized coating antibody and the enzyme-linked polyclonal antibody specific for IL-8. Colour development then allows measurement of the intensity to allow quantification.

2.4.3 IGFBP-1 ELISA

IGFBP-1 was measured by a two-site sandwich ELISA with matched pairs of capture and biotinylated detection antibodies for IGFBP-1. Diluent used was PBS. The IGFBP-1 capture antibody (100µl/well) was added at 2µg/ml, and plates prepared as in the IL-8 ELISA method.

The plates were washed prior to use and a standard curve (4ng/ml to 0.06ng/ml) added in single wells to each plate. 100µl NSB and quality control wells (at 700pg/ml) were added in quadruplicate. Samples (100µl) were added in duplicate, sealed and incubated at room temperature on an orbital shaker for 2 hours. After washing (x4) and tapping dry, detection antibody (0.1µg/ml; 100µl/well) was added for a further 2 hour incubation on the plate shaker. After further wash and dry cycle as before, streptavidin peroxidase conjugate (0.125U/ml; 100µl/well) was added, to incubate for 20 minutes, before washing and developing with substrate (100µl/well) and quenching with 2N sulphuric acid (50µl/well). The optical density was then determined by a plate reader set at 450nm. The computer programme AssayZap was used to analyse the results and construct a standard curve against which the samples were then compared. The within assay precision was 1.7% (relative standard deviation) and all assays were analysed on the same run.

2.4.4 Prolactin Fluoroimmuonassay

A Wallac DELFIA prolactin kit (Perkin Elmer™ Life Sciences) was used to assay prolactin release in culture medium. The kit is designed for use in human serum and is a solid phase, two-site fluoroimmunometric assay based on the direct sandwich technique. The two antibodies are directed against two separate antigenic sites on the hPRL molecule, the first immobilises the sample hPRL while the other is europium-labelled. Enhancement solution dissociates europium ions from the labelled antibody into solution and forms fluorescent chelates with them. The fluorescence measured is proportional to the hPRL concentration in the tested sample.

Reagents and samples were brought to room temperature before use. A 96 well plate, pre-coated with solid phase anti-hPRL IgG is supplied with each kit. Europium-labelled antibody (mouse monoclonal) at 200µl/well was added, and then standards and samples were added in duplicate. Standards are supplied in the kit and range from 0-250ng/ml. Expected concentrations in culture medium were at the lower end of these standards so sample volume was multiplied by a factor of eight and results then divided accordingly. A 90 minute-incubation at room temperature, on orbital shaker was followed by washing prior to the addition of enhancement solution (200µl/well) and shaking for 5 minutes. Fluorescence is then measured within 30 minutes on a time-resolved fluorometer (VICTOR).

The analytical sensitivity (the value which is 2 standard deviations above the mean of the zero standard measurement values) is reported as "typically better than 0.04ng/ml". The within assay variation was 7.5% (r.s.d) and all samples were analysed on the same run.

2.5 FLUORESCENCE ACTIVATED CELL SORTER (FACS)

This method is based on flow cytometry (Coulter ® EPICS ® XL^{TM}). Flow cytometry is used to optically quantitate components or structural features of cells, which are individually counted but rapidly processed so thousands of cells can be measured in seconds. The cells are passed through a laser beam as single cells in suspension, and registered as events. The light then scatters; forward scatter is related to cell size and side scatter relates to cell granularity. Cells also emit fluorescent light when excited by the laser. Different probes, which fluoresce at different wavelengths, can be used to quantify specific components of cells recognised by labelled antibodies. The three measurements of cell size, granularity and fluorescence combine to illustrate the distinct populations of cells present and the percentage of fluorescent gives the percentage of antigen–positive cells in a sample.

2.5.1 Fibroblast Cell Purity

Human cervical stromal cell cultures were assessed for purity with a human monocloncal anti-fibroblast antibody Ab-1 (Oncogene).

Cultured cells were trypsinised and resuspended in medium before being split into three 1.5ml eppendorf tubes. To one tube 2µl fibroblast antibody was added and all three tubes were placed on ice for 30 minutes. Tubes were then spun at 4000rpm for 1 minute to pellet and resuspended in CSM (cell separation medium). Two further washes were performed. To two tubes, 100µl CSM and 10µl anti-mouse IgG whole molecule with FITC conjugated (Sigma) was added and then cells re-suspended. The remaining tube was a blank. The tube exposed only to the anti-mouse IgG is the negative isotype-matched control. Tubes wrapped in foil to prevent light exposure and kept on ice for 30 minutes. Tubes washed as before and re-suspended in 500µl FACS buffer. The cells were the passed through cell strainers (Becton Dickinson Labware Europe, France) to ensure single cell suspension, and the passed through the FACS machine.

A typical FACS report is shown is shown in Figure 2.5

Figure 2.5 *A representative example of a FACS report for the fibroblast purity of cultured cervical stromal cells*

A) Isotype-matched negative control

B) Sample with fibroblast antibody

The y axis is log of forward scatter and x axis is the log of side scatter.

Graph i) total no. of counts is illustrated and the region within the green line represents the gated area of cell analysis. Remaining points outside this are likely to be cell debris and are excluded from analysis.

Graph ii) the y axis represents the total cell count and the x axis is FITC value - the total level of fluorescence detected within the gated region in i). The red line indicates those positive for FITC.

Note the FITC value is minimal in A) the negative control, but in B) where the sample has been incubated with the fibroblast antibody the majority of cells are positive for FITC.

A

MRC EDINBURGH

COULTER(R) EPICS(R) Acquisition Flow Cytometry Report

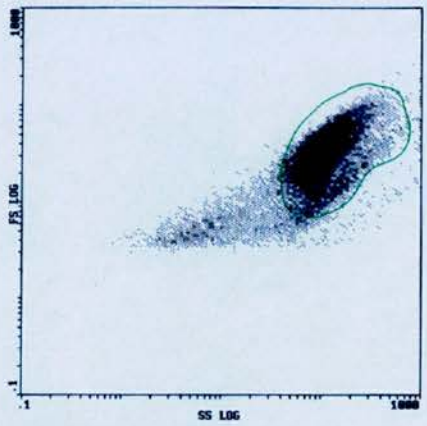
11Feb03 16:00:27
Shona fitc fibo 11.02.03
Z0014972
S.C. 11.2.03 4a
66 seconds. 11767 events
Stop Count: 10000 events. histogram 3

DP ID: SJD

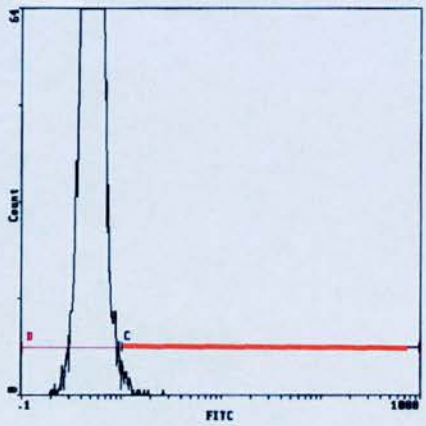
CT006 -ve

Initial cytosett. from prot. #1 FITC 8.10.02

i)



ii)



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	85.0	10000	110.7	28.2	88.7	30.1	55	51.73	59.65

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
3	C	C	1.19	119	1.39	1.20	1.04	8	1.07	1.04	1024
	D	D	100	10000	0.525	0.522	0.561	214	13.82	0.102	996.7

B

MRC EDINBURGH

OULTER(R) EPICS(R) Acquisition Flow Cytometry Report

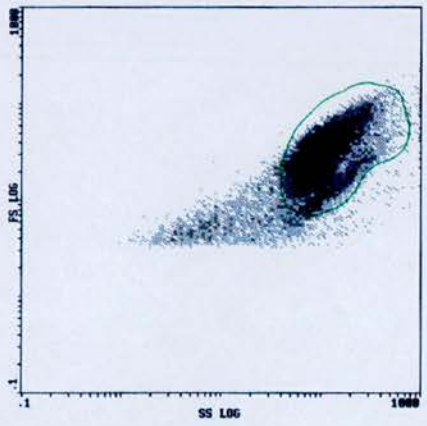
11Feb03 16:02:37
Shona fitc fibo 11.02.03
Z0014974
S.C. 11.2.03 4b
71 seconds. 11883 events
Stop Count: 10000 events. histogram 3

P ID: SJD

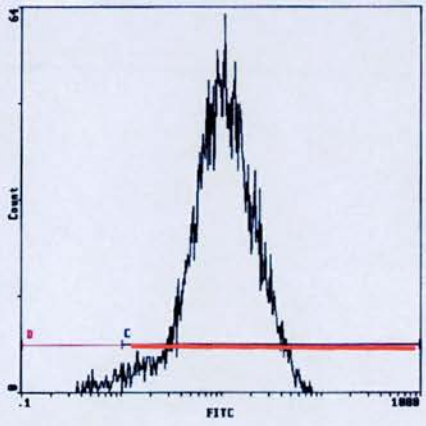
CT006 +ve

itial cytosett. from prot. #1 FITC 8.10.02

i)



ii)



Stats: Normalized, Listgating: Disabled

Color equations

Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	84.2	10000	111.3	27.4	82.5	32.4	46	54.58	60.08

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
3	C	C	98.0	9795	10.6	10.8	11.0	70	23.32	1.04	1024
	D	D	100	10000	9.99	10.6	11.0	70	23.32	0.102	996.7

2.5.2 CD10 expression

Cells were trypsinised and resuspended in cell separation medium to achieve a cell density of 4-8 x 10^5/ml. This suspension was then centrifuged at 4000rpm for 1 minute to form a pellet before resuspension in FACS buffer (sterile PBS with 1%FCS and 0.1% azide). A repeat spin was followed by resuspension in 100µl of FACS buffer. To this 20µl of CD10 phycoerythrin labelled probe (human anti mouse) was added and incubated for 30 minutes on ice and protected from light. A blank and an isotype (mouse IgG) control was also prepared for each cell line. For each treatment there were 3 vials:

1. Control
2. Isotype control labelled
3. CD10 phycoerythrin labelled probe

All samples were then washed, spun and resuspended three times in FACS buffer to a final volume of 1ml. Cells were then analysed by flow cytometry in a Beckman Coulter FACScan. Fluorescence intensity was then plotted against number of events.

Statistical analysis

Significant differences of PCR and ELISA results were determined by analysis of variance (ANOVA; Statview 3.0).

Data normalised to control was analysed by non-parametric methods. The Kruskal-Wallis analysis of variance was used and Dunn's multiple comparisons test was used to assign significance to treatment. For comparisons of RNA in pregnant or non-pregnant cervical biopsies, the Mann-Whitney U test was used.

Statistical differences are indicated on graphs by the use of symbols above the relevant bars. P values relating to the symbols used are detailed in the figure legends. $P<0.05$ was taken as being significant.

CHAPTER 3

NEEDLE-FREE INJECTOR AS A CERVICAL DRUG DELIVERY SYSTEM

3.1 INTRODUCTION

Cervical ripening is essential to the normal progress of labour. It is known that approximately 20 % of pregnancies in the UK currently undergo some therapeutic intervention to initiate labour. Prostaglandins are the most commonly used agents, currently administered vaginally or intracervically in the form of a gel, tablet or hydrogel polymer. This method relies on variable drug absorption and can have significant side effects. A needleless injector may provide a route of administration whereby a precise dose can be given directly to the target tissue producing a more rapid and controlled response.

Cervical ripening is a complex process culminating in the softening and restructuring of the cervical connective tissue in preparation for cervical dilatation throughout the process of labour. There are many mediators involved in this but the most therapeutically exploited to date are prostaglandins. Prostaglandins of the E and F groups are known to have a physiological role (Gibb 1998) and have been used successfully for cervical ripening, not only for labour but also for termination of pregnancy and other procedures requiring access to the uterine cavity (Calder *et al* 1993).

Prostaglandins E and F do not only affect cervical tissue but also cause the myometrium to contract and this is one of the major concerns, as it occasionally results in hyperstimulation. Rarely the catastrophic complication of uterine rupture may occur (Bennett 1997). In fact, when given systemically, prostaglandins can produce gastro-intestinal, respiratory and cardiovascular side effects and this is one of the reasons that intravenous, oral, extra-amniotic and intramuscular use now is largely avoided.

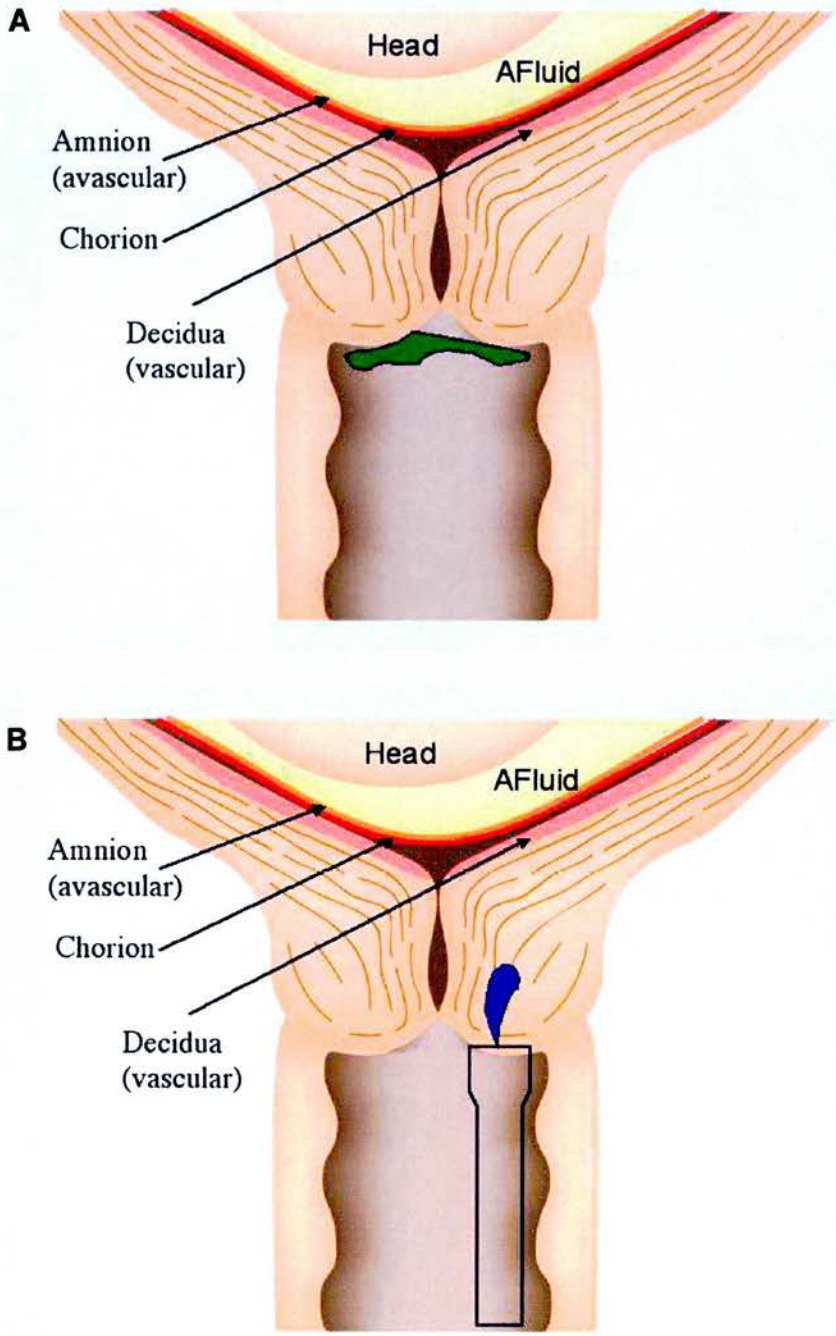
The current favoured agent is dinoprostone (PGE_2), but many formulations require special storage and cost is a limiting factor in developing countries. With pharmaceutical developments, local administration became far more popular

reducing the side effect profile significantly and vaginal/ intracervical gels /tablets and vaginally administered hydrogel polymers are now widely used (Keirse 1992; Calder *et al* 1993; Arias 2000). There is little to choose between these formulations in terms of efficacy, but patient satisfaction scores were slightly better for the hydrogel polymer which is left in-situ for up to 12hrs, when compared with vaginal gel where repeat examinations and drug administration may be required within that time (Tomlinson *et al* 2001). However the improved satisfaction scores related to labour rather than the induction process.

As knowledge of the cervical ripening process continues to expand, so therapeutic options may increase. Drug delivery for this purpose is felt to be more appropriate locally, as systemic administration, particularly of prostaglandins, which are most commonly used in this scenario, can cause significant side effects. Currently we rely on absorption through the vaginal/ cervical epithelium for any drug to take effect and this results in administration of an excessive dose to achieve absorption of the required amount. It is increasingly suggested that the internal cervical os is the site of initiation of cervical ripening (Van Meir *et al* 1997; Challis *et al* 1999) and is notoriously difficult to target, due to the anatomy and the very dense structure of the cervix. It is considered that by bypassing the absorption requirements, a more direct delivery may allow lower dosage administration and may achieve a more rapid response. The needleless injector form of drug delivery is currently used for subcutaneous and intradermal administration (usually of hormones eg. growth hormone and insulin), and may provide an attractive alternative to current cervical ripening therapeutics.

Figure 3.1 ***Anatomical features with local drug delivery***

A) vaginal gel B) needleless intracervical injection



Hypothesis

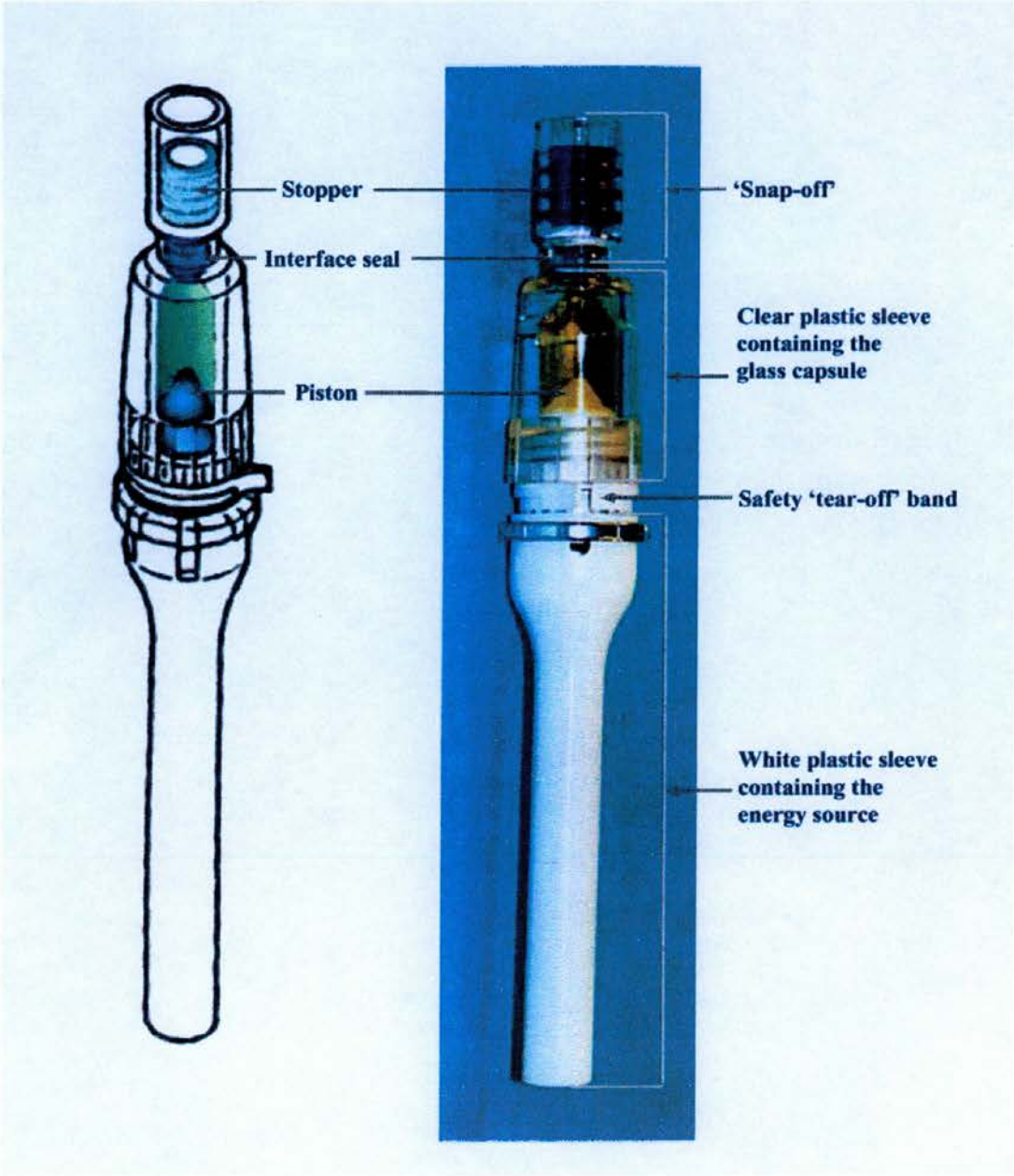
The needleless injector has been used to deliver compounds both subcutaneously and intradermally. The cervical stroma is the target tissue for cervical ripening agents. One problem in drug administration to the cervix is the stratified squamous epithelium through which diffusion is relatively slow, and another is the dense nature of the fibrous cervix. Absorption after vaginal application is variable and requires excess drug to be administered to achieve the desired effect. Some compounds cannot be delivered in this manner due to molecular size or hydrophilic nature. In these cases it would be desirable to bypass the epithelial barrier, by injection technique, and it is supposed that avoidance of needle use is attractive for users. In addition the diffuse distribution of drugs using injection of a liquid stream may be beneficial for subsequent diffusion. This form of delivery system has not been assessed with cervical tissue and so this study aimed at preliminary assessment of suitability. Does the needleless injector breach the cervical epithelium and penetrate the cervical stroma?

Needle free injector device

The device uses a compact nitrogen gas source to propel a quantity of liquid through a tiny aperture (0.3mm), thereby breaching the epithelial barrier, and penetrating the underlying tissue. There is a gas spring and trigger mechanism and a capsule containing the liquid. Once the safety band is removed, pressure is applied against the target until the trigger latch disengages. On activation the ram is pushed forward by the compressed gas. This pushes the piston down the drug capsule, expelling the solution in less than 60 milliseconds.

Needle free injector devices have been successfully used in dental practice and for self administration of subcutaneous insulin. Differing designs are on offer (reusable or disposable) and compression gases vary (eg. CO_2) have been used.
(See www.syrijetinc.com/public/intro.html or www.jtip.com/luersys.htm)

Figure 3.2 ***Components of the needle-free injector device***



Weston needle-free injection device

The Weston intraject device is a single use, disposable, needle-free injection system for the delivery of liquid drugs. It uses compressed nitrogen as an energy source and once filled, delivers a pre-measured quantity of drug to the site chosen. The liquid drug is held in a glass capsule contained within an outer clear plastic sleeve. The device cannot be used on a second occasion as there is no residual energy after use. This also means that the device can be easily and safely disposed of as there is no needle and no residual energy.

Aims:

1. To assess a needleless injector device as a delivery system to the cervix.

2. To measure depth of penetration of dye administered by a needleless injector.

3. To obtain histological evidence of dye penetration.

4. To obtain histological information about degree of tissue trauma.

3.1 METHODS

Uteri were obtained as described in chapter 2. Six cervices were used in the study with a proposed two injections per cervix. See details in table below. All women were undergoing surgery for benign disease and were pre-menopausal.

Table 3.1

	Age	Parity	Vaginal:caesarean delivery	Operation
1	32	2	1:1	TAH
2	37	2	2:0	TAH
3	32	4	4:0	TAH
4	31	2	2:0	VH
5	45	2	2:0	TAH
6	37	3	3:0	LAVH
	35.7 yrs (Mean)	3 (Median)		

The needle-free injector devices (Weston Medical Limited, UK) were filled prior to use with 0.2mls methylene blue dye (1mg/ml) in sterile phosphate buffered saline. Each uterus and cervix was collected from theatre and injected within 30 minutes of removal from the subject. The site of injection was the ectocervix of the anterior and posterior cervical lip with the direction of injection towards the uterine body. After each injection a coronal or sagittal incision was made, measurements performed and photographs taken (see Figs 3.3-3.6). The camera used was Nikon D4 with a Tamron macro lens. Each picture contained a scale in millimetres. The main outcome measure was the mean depth and lateral spread of dye injected. Measurements of depth and lateral spread included any evidence of blue dye spread from the site of injection, both intense and lesser degree of staining.

After all measurements were recorded and photographs completed each sample was returned to theatre, and placed in formalin. After overnight fixation, specimens were examined macroscopically, dissected, and tissue blocks taken from the cervix and processed by routine methods. Haematoxylin and eosin stained sections were made and examined microscopically. In addition, unstained sections were mounted on glass slides and examined microscopically for traces of dye.

The last sample received one injection from a needle-free injector device and one from a conventional needle and syringe filled to the same volume. It was felt that depth of injection cannot be compared as the needle must penetrate the tissue to some degree before injection thereby falsely increasing the measurement of depth of spread. This was to allow a comparison of the pattern of distribution i.e. is there a more diffuse pattern of distribution with a needle-free injector reflected by a greater degree of lateral spread? All the photographs shown represent the needle-free injection method.

Figure 3.3 *Cervical sample showing sections taken*

Figure 3.4 *Coronal section showing dye distribution (all scales in millimetres)*

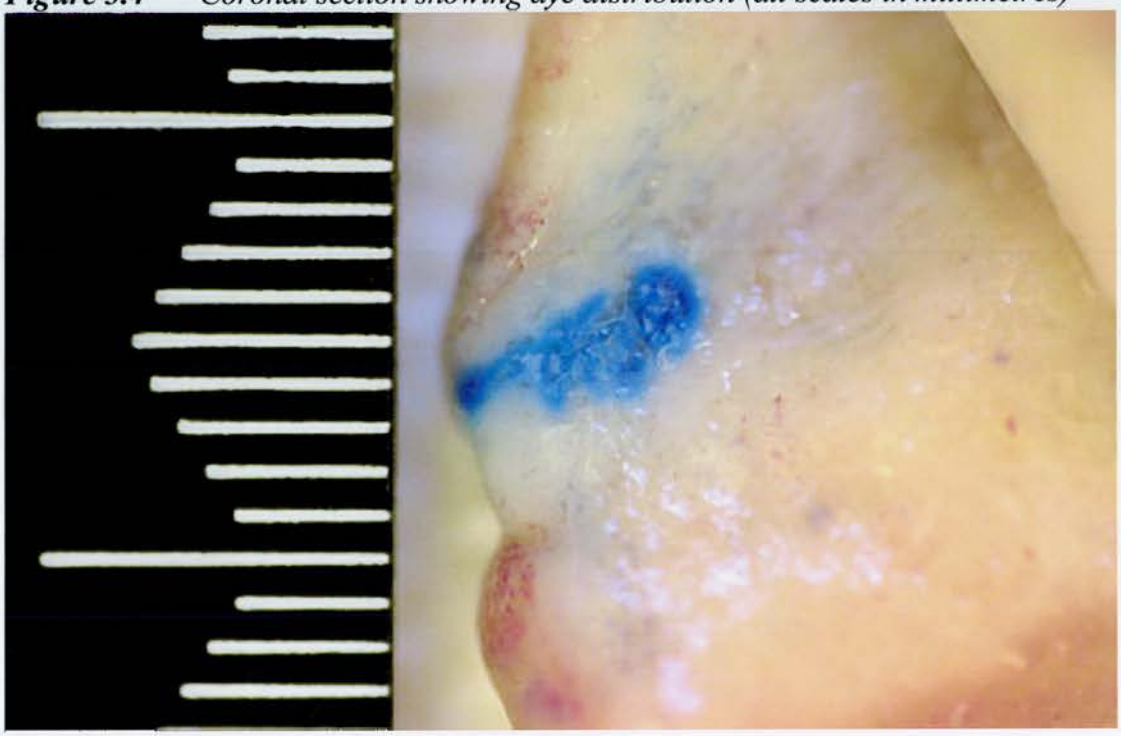


Figure 3.5 *Sagittal section showing dye distribution*

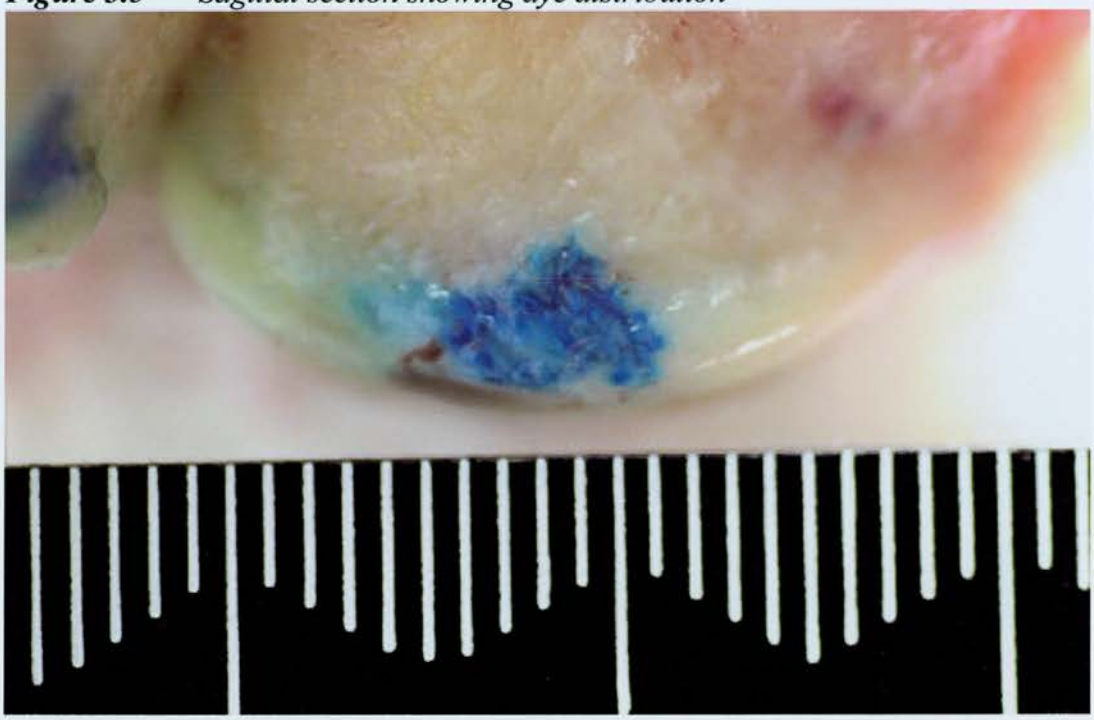
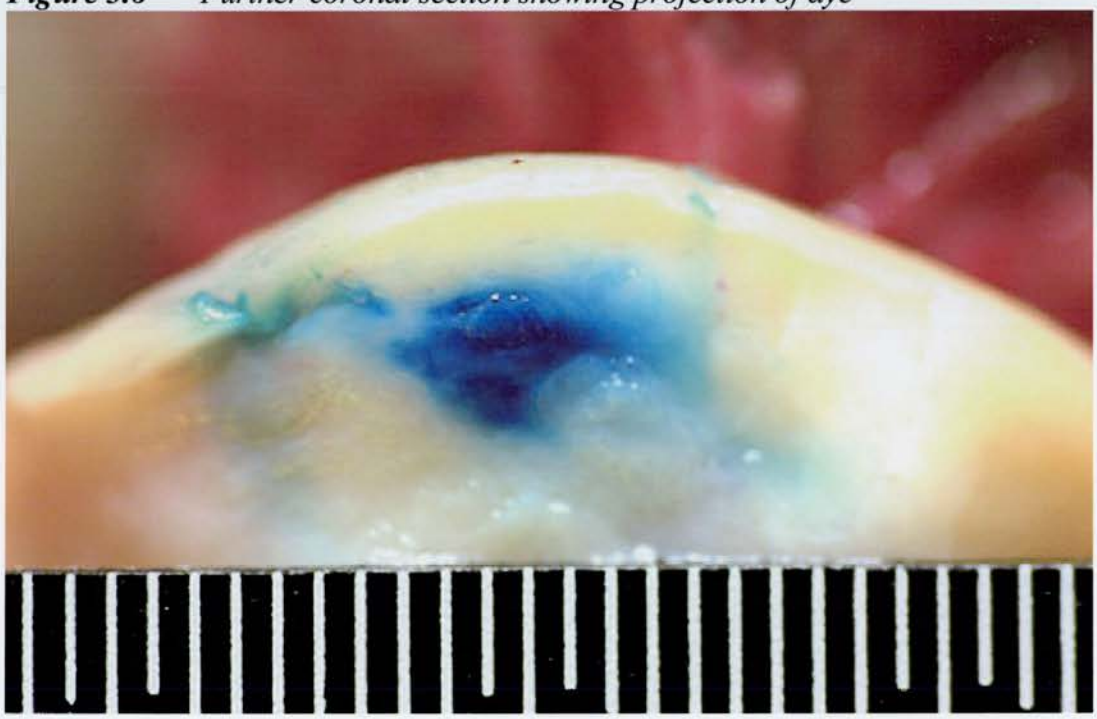


Figure 3.6 *Further coronal section showing projection of dye*



3.2 RESULTS

The results are shown in the tables below (Tables 3.2 & 3.3). The needleless injector penetrated a mean depth of 5.6mm with a mean lateral spread of 10.3mm. This is sufficient to breach the cervical epithelium and deliver an agent directly to the cervical connective tissue. This would avoid the problem of variable absorption in vaginally delivered gels. One sample was taken at the time of vaginal hysterectomy, the indication for the procedure being uterocervical prolapse, which is felt to be associated will some form of collagen dysfunction. This sample resulted in the greatest measurements for depth of penetration and may be related to deficient collagen and lower tensile strength of the tissue. If this is the case one might assume that greater penetration may occur in pregnant cervices as the process of cervical connective tissue remodelling begins early in pregnancy and accelerates in the final few weeks.

Pathology

After routine processing for histology, no trace of dye was seen microscopically in any of the six specimens, neither on haematoxylin and eosin-stained sections, nor on the unstained sections of cervix. It is believed that the dye dispersed during processing. However, in none of the specimens was there any evidence of tissue trauma secondary to the injection procedure. The use of an alternative dye that persists throughout fixing and routine preparation for microscopy would have been beneficial and corroborated the gross findings and given us a more accurate assessment of distribution. Suggested alternatives might be toluidine blue or india ink.

Table 3.2 *Depth of penetration*

Sample	Depth (mm)	Mean/sample	Mean	s.e.m.	Median
1	2.5	2.75			
	3				
2	5	5.25			
	5.5				
3	3	3.5	5.59	1.4	4
	4				
4	7	13			
	19				
5	4.5	4.25			
	4				
6	4	4			

Table 3.3 *Lateral spread*

Sample	Lateral spread (mm)	Mean	s.e.m.	Median
1	6			
2	9			
3	7	10.3	1.4	11
4	13			
5	14			
6	13			

Needle comparison: depth 16mm, lateral spread 8mm.

3.4 DISCUSSION

These findings support the hypothesis that needle-free injectors breach cervical epithelium when used on ex-vivo uteri. This may warrant further investigation for the application of drug delivery to the cervix. Initial assessment confirms that this delivery system would penetrate the cervical epithelium, thereby reaching the target immediately and possibly shortening the duration of the ripening process. This would also enable more accurate dosage regimes and as a local delivery system, minimise systemic side effects of any proposed agent. Not only might his be used for cervical ripening agents but also to administer local anaesthetic agents for colposcopic or other gynaecologic procedures.

Cervical ripening is the preparatory connective tissue remodelling prior to labour to enable the cervix to dilate. The cervix is a very dense fibrous structure out with pregnancy (Danforth 1947) and through the majority of pregnancy, but there is a gradual change in cervical connective tissue which accelerates immediately prior to labour (Ekman *et al* 1986). The end result of this process is a remodelled structure with reduced collagen content, increased water content and a change in relative quantities of different glycosaminoglycans. This then causes disassociation of the collagen fibrils, and with a neutrophil influx, an increase in degradative enzyme activity.

Cervical fibroblasts may be central to the ripening process. There is now interest in several other inflammatory mediators thought to have some role in cervical ripening (Sennstrom *et al* 2000). The premise here is that leukocytes are recruited to the cervix by some inflammatory mediator released from the fibroblast. The activated leukocytes then participate in the inflammatory process of ripening by releasing collagenases and matrix metalloproteinases, which in turn remodel the connective tissue resulting in reduced collagen content, increased water content and a softer cervix (Liggins 1981; Romero *et al* 1991; Ledingham *et al* 1999a).

Whatever agent is considered for cervical ripening there may be significant benefits in the proposed delivery method, ideally to promote cervical ripening without inducing myometrial activity until this process is complete. The attraction of delivering the required compound directly to the cervical connective tissue (largely collagen fibrils and fibroblasts), thereby avoiding variable absorption rates with unpredictable responses, and acting directly on the target tissue is considerable.

The use of an alternative dye that persists throughout fixing and routine preparation for microscopy would have been beneficial, corroborated the gross findings and given us a more accurate assessment of distribution. This was an unintentional yet significant short-fall in this study and I suggest that more appropriate dyes might be toluidine blue or india ink.

Having used these devices on postoperative organs in non-clinical set-up it would be important to trial their use in-vivo without active agent, largely to assess practical issues such as device trigger pressure, ease of use, and user and patient acceptability. The needle-free injector may provide efficient drug delivery to the cervix for gynaecological outpatient procedures as well as induction of labour and pre-surgical termination of pregnancy cervical preparation. There are considerations in device design to consider, such the trigger pressure required to fire the device. Currently this requires forward pressure against the chosen target until a threshold is reached that automatically fires the gas release. Perhaps this threshold pressure would need alteration for particular tissue targets or the incorporation of a manual trigger would be beneficial. A threshold pressure set too low would be potentially dangerous if triggered before correct placement, and too high would be impractical for clinical use as the cervix would require to be immobilised. This would cause further discomfort and negate the potential benefit of needle-free injection. However, significant design alterations were not an option in these circumstances as a single device design was available. Also because of the anatomical site of the cervix, the existing device would require an extension to allow use with a speculum. With good visualisation of the cervix a simple lengthening of the handle would suffice and it is unlikely that introduction of an angle along the handle would be a significant improvement. The

sound of gas release with firing the device is something that cannot be prevented and with adequate pre-procedure counselling is unlikely to be a handicap.

In summary this shows the needle-free injector delivery system is effective in traversing the cervical epithelial barrier and may therefore successfully deliver compounds unsuitable for other methods of administration. This study confirms the viability of use of such a device with regard to tissue penetration, but it remains to be seen whether this would offer a clinical benefit. The anticipated benefit would be accurate, reliable and anticipated reduced drug dosing. A potential benefit would be speed of subsequent distribution with a shorter interval to effect. An improved efficiency of any drug delivery method for labour induction could shorten the process for those that currently require repeated administration of vaginal gel over 24-48 hours.

To assess clinical benefit of a needle-free injector, a trial would require comparison against standard drug delivery methods (i.e. vaginal gel). Initial further assessment would require placebo use of the device in women to assess acceptability and safety. It was my intention to proceed to this but several significant delays were encountered postponing tissue collection in the first instance and later device supply was withdrawn as re-design issues were dealt with. Initial delays were awaiting Ethics Committee and Trust Management Approval. Although applications were submitted early and there were no concerns with study design, I encountered significant delay as a result of legal discussions regarding Intellectual Property Rights and publication issues between the Trust R&D, University department and Ardana Biosciences. As this was the first such collaboration locally there were unforeseen issues that required clarification and communication between the relative departments was laboured. In addition to these delays, subsequent postponement of research activity was inflicted as the clinical department, and later the research unit were relocated to a new site causing major disruption and down-time on each occasion. As a result the subsequent plans for a clinical assessment study were abandoned and further work concentrated on in-vitro cervical fibroblast studies.

There is no evidence to suggest that this method would be preferable than injection with a dental syringe as is routinely used in colposcopic practice and both these methods require lithotomy positioning, speculum examination and good lighting. Either injection technique is much more cumbersome than current induction procedures where a simple vaginal examination is suffice, but while practical, the inadequacies of current induction techniques should not be ignored. Only if the induction process were sufficiently improved in terms of duration from induction to delivery or outcomes would this more invasive method seem justified.

At present optimal dosage studies of current vaginally administered induction agents are in progress and may offer significant improvement in terms of safety and efficiency. Although this device offers theoretical benefit for cervical ripening drug delivery, I have several concerns about the practicalities in clinical use and therefore I do not feel that further investigation is merited. With regard to outpatient gynaecological procedures the examination procedure would not change but there are minimal difficulties with dental syringe use and so little to be gained with the added expense of device purchase.

CHAPTER 4

DECIDUALISATION OF CERVICAL STROMAL CELLS

4.1 INTRODUCTION

Although the uterus and cervix have distinct anatomy and disparate roles they do co-exist in physical continuity with similar exposures. The process of endometrial decidualisation is essential for successful pregnancy and this transformation of the endometrial stromal cell is dependent upon progesterone response mediated by the progesterone receptor. The capacity for the cervical stromal cell to respond to decidual stimuli in a manner similar to it's endometrial counterpart has not been explored despite previous identification of decidual-like cells in the human cervix (Rutanen *et al* 1991). Decidualisation follows activation of a series of progesterone-dependent gene sets (Tabanelli *et al* 1992). This provides a window of opportunity for blastocyst implantation and trophoblast invasion. In the absence of pregnancy falling progesterone levels leads to vascular disruption and the superficial endometrium is shed.

Previous *in vitro* studies in decidualisation conclude that progesterone is critical and that cAMP augments the response via sustained protein kinase A activation (Brar *et al* 1997; Telgmann *et al* 1997; Gellersen *et al* 2003). Although in laboratory practice this involves treating with cAMP analogue, as cAMP does not cross cell membranes with ease, this could be achieved through various mechanisms physiologically either via the PGE_2 receptor pathway or by alteration of cAMP synthesis/ catabolism. An example would be to influence phosphodiesterase activity, with specific PDE inhibitors or with relaxin, which has also been shown to have this effect (Bartsch *et al* 2001). EP2 is known to be the dominant PGE receptor in pregnant baboon cervices (Smith *et al* 1998) and provides a mechanism of cAMP regulation via the EP2 receptor/ PGE_2 pathway (Brar *et al* 1997). *In-vitro* endometrial decidualisation studies traditionally use a decidualisation mix including progesterone, cAMP and oestradiol based on the observation *in-vivo* that the progesterone response necessitates oestradiol-primed stroma (Huang *et al* 1987; Telgmann *et al* 1998). The dramatic effect seen with progesterone and cAMP combined confirms their critical

role. An oestradiol-specific effect is not apparent from *in-vitro* studies and questions the need for oestradiol in these models.

Known decidualisation markers from the body of work on human endometrium are many but the most reliable are PRL and IGFBP-1 (Tabanelli *et al* 1992; Lane *et al* 1994; Brar *et al* 2001). The precise function of the decidualised cell is still undetermined but key functions would appear to include regulation of extracellular organisation, cell migration, angiogenesis and haemostasis. Decidual IGFBP-1 has a high affinity for IGFs altering their bioavailability and thereby inhibiting local actions (Lane *et al* 1994; Gibson *et al* 2001).

Endometrial stromal cells express both PR isoforms (Brosens *et al* 1999) and are sensitised to PR-mediated progesterone effects by raised cAMP levels and sustained PKA activation. Persistently elevated PR levels inhibit decidualisation (Brosens *et al* 1999). Prolonged progesterone exposure downregulates PR reversing PR-mediated IGFBP-1 inhibition, possibly limiting decidual phenotype expression. PR isoforms are also present within the human cervix and so phenotypically similar cervical cells have the potential to respond in a similar manner (Stjernholm *et al* 1997; Stjernholm *et al* 1999).

Decidua has a basement membrane-like structure with increased intercellular space rich in matrix proteins such as fibronectin, laminin and collagen. These upregulated matrix proteins interact with placental integrin receptors forming the fetomaternal interface. Fibronectin is thought to have a particular role in cell migration during implantation, embryo and fetal development (Duband *et al* 1990), and is upregulated by progesterone via the PR (Zhu *et al* 1992; Brar *et al* 2001; Tseng *et al* 2003). Laminin, desmin and tissue factor are other identified decidual products (Tabanelli *et al* 1992; Lockwood *et al* 1993; Brar *et al* 2001). Desmin, typically expressed by myofibroblasts, is not seen in decidual precursors but has been identified in DSC (Glasser *et al* 1986; Oliver *et al* 1999). Human DSC also express CD10 antigen, an endopeptidase associated with haematopoietic cells, yet ultrastructural studies concur

with the mesenchymal origin of DSC (Montes *et al* 1996; Oliver *et al* 1999; Kimatrai *et al* 2003).

This experimental work aims to establish whether there is evidence of decidual-like changes within the human cervix in early pregnancy, by assessment of decidualisation marker mRNA expression, primarily prolactin and IGFBP-1. In view of the limited availability of cervical whole tissue, particularly in pregnancy, a proposed model is suggested to evaluate the cervical fibroblast/ stromal cell and its response to decidual stimuli. Endometrial decidualisation evolves from the perivascular stromal cell (Kelly *et al* 2002) and so, should a similar mechanism exist within the cervix, the fibroblast is a likely contender. The fibroblast is a cell of multiple functions and may prove to be central in orchestration of the events seen throughout pregnancy and labour. It is of particular interest to explore its response to a pro-decidualisation environment. Phenotypic alteration would have significant implications for the subsequent functions of this cell type.

It is critical to compare the influence of progesterone and cAMP individually in this process, and whether a combined approach demonstrates true synergism as is suggested from the endometrial data. Pregnancy-derived and non-pregnancy derived cervical samples and stromal cells are compared in their response to decidual stimuli.

Decidual-like transformation may be an inherent property of all fibroblasts with appropriate stimulation. A comparison will be made with a non-reproductive cell line (foreskin fibroblasts) on the assumption that this capacity is peculiar to reproductive tissues in possession of nuclear progesterone receptors.

Hypothesis

This work aims to prove the hypothesis that human cervical stromal cells undergo a phenotypic alteration akin to decidualisation in pregnancy and that this can be induced *in-vitro* by a combination of progesterone and cAMP.

Aims:

1. To compare gene expression of decidualisation markers in pregnant and non-pregnant cervical biopsies.

2. To apply decidual stimuli *in vitro* to cervical stromal cells from both pregnant and non-pregnant women and compare the response in terms of;
   a) Morphology
   b) Gene expression of markers of decidualisation
   c) Protein release of decidualisation markers
   d) Receptor status
   e) Surface marker expression

3. To compare cervical stromal cell and foreskin fibroblast response to decidual stimuli.

4.2 METHODS

Tissue collection

Cervical stromal cells were isolated from both non-pregnant (n=6) and pregnant (n=7) cervices as described in chapter 2.

A foreskin fibroblast cell line (HS27 obtained from the European Collection of Cell Culture) was used to compare the decidualisation response in a non-reproductive fibroblast cell line.

Cervical biopsy mRNA studies

Both pregnant and non-pregnant samples were homogenised in Tri reagent for RNA extraction from untreated cervical biopsy tissue.

Tissue culture/ storage

All cervical fibroblasts were cultured as described in Chapter 2. Cells used were from passage 2-7.

HS27 cells are foreskin fibroblasts and were used as a non-reproductive cell comparison. These cells were supplied at passage 23, were incubated in an identical manner as in complete medium and treated at passage 24-25.

Treatment regimes

All cells were plated at 5 x 10^4/ ml complete medium in 6 well plates and grown to confluence over 72hrs before treatment commenced. Following a PBS wash treatments were prepared with 2% FCS supplemented RPMI (with 20μg/ml gentamicin, 100IU/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine):

1. Control
2. 8-bromo-cAMP 250μM (cAMP)
3. Medroxyprogesterone acetate 10^{-6}M (MPA)
4. Decidualising Mix (8-bromo-cAMP 250μM + MPA 10^{-6}M + oestradiol 10^{-7}M)

The combined treatment of MPA, cAMP and oestradiol will be referred to as decidualisation mix (DM). Treatment periods were for 6 or 10 days, with medium changes every 3 or 4 days (pregnant cell lines n=5, non-pregnant n=6). Medium was collected in duplicate at the end of each treatment period and stored at -20°C for protein assay at a later date.

Decidualisation mix containing cAMP, MPA and oestradiol was used based on previous experience in the local laboratory with endometrial decidualisation. Oestradiol could therefore have a confounding effect as this was not compared alone. In view of the potential confounding factor of oestradiol, a comparison experiment was performed using Decidualising Mix with and without oestradiol (n=4):

1. Control
2. 8-bromo-cAMP and MPA
3. DM (8-bromo-cAMP 250μM, MPA 10^{-6}M and oestradiol 10^{-7}M)

Real time-PCR

RNA extraction was performed using Tri Reagent and Phase-lock tubes according to detail in Chapter 2, followed by precipitation with isopropanol. All RNA samples were quantified by UV spectrophotometry before dilution to 100ng/μl. Generally, 4μg of total RNA was reverse transcribed, and the resulting single-stranded cDNA was then analysed by quantitative, real time, Taqman PCR using a Model 7700 Sequence Detector.

Primers and probes were designed using the Primer Express© program (Applied Biosystems). Sequences used in are shown in Table 2.1.

All PCR reactions were run in duplicate, with 18-S as an internal endogenous control to normalise variations in cDNA content between samples. To determine genomic DNA contamination the β-actin signal was measured without reverse transcription in all samples. The criterion for exclusion was a measurement greater than 3 standard deviations below the mean, from a population of 66 samples; this translates to a β-actin FAM signal with a Ct below 27 cycles. Samples used had a mean β-actin FAM Ct of 34.6 ± 0.4 and the minimum was 29.1.

To examine the gene expression of known decidualisation markers in untreated cervical biopsies, RT-PCR was carried out for PRL, IGFBP-1, laminin, fibronectin and VEGF. Phosphodiesterase type 4 was also measured as a potential marker for physiological cAMP regulation. Receptor expression status was evaluated for progesterone receptor (PR) prostaglandin receptor types EP2 and EP4.

Where cultured CSC were treated, decidualisation markers PRL and IGFBP-1 mRNA expression was compared with control. In these groups progesterone, EP2 and EP4 receptor expression was evaluated.

Enzyme Linked Immunosorbent Assays

Secretion of PRL and IGFBP-1 proteins were evaluated in medium collected at the end of each treatment period. All samples assayed in duplicate. PRL estimation was performed using a PRL kit as described in Chapter 2 but with a 10-fold increase in sample volume relative to standard volume, corrected during analysis. The analytical sensitivity is 40pg/ml and the within assay variation was 7.5% (relative standard deviation). All samples were analysed in the same assay.

The IGFBP-1 assay was performed as described in Chapter 2 with a lower limit of sensitivity of 62.4pg/ml and a within assay variation of 1.7% (relative standard deviation). Again, all samples from each experiment were analysed on the same assay.

Fluorescence Activated Cell Analysis

FACS for anti-fibroblast antibody: for details see Chapter 2

Cervical stromal cells were grown to confluence and treated with trypsin EDTA, washed and resuspended in PBS/BSA medium. The cell suspension was treated with monoclonal anti-fibroblast antibody (Oncogene Research Products), which recognises a fibroblast specific surface antigen. Cells were then washed and treated with fluorescein labelled sheep-anti-mouse serum. Cells were analysed in a Beckman Coulter instrument and fluorescence intensity was plotted against number of events.

FACS for CD10 surface marker: for details see Chapter 2

A repeat experiment was run for 10 days comparing control pregnant (n=2) and non-pregnant (n=2) cervical stromal cells, to those treated with decidualisation mix as above (DM).

4.3 RESULTS

4.3.1 Cervical biopsy data

Cervical biopsy samples from pregnant and non-pregnant women were homogenised and RNA extraction performed. Due to limited tissue samples RT-PCR was performed on pooled RNA for some markers (PRL and IGFBP-1), and individually for others with a mean and s.e.m calculated (range shown in table 4.1).

PRL and IGFBP-1 mRNA were both found to be raised in pregnant samples relative to non-pregnant samples (14-fold and 53-fold respectively), but this was on pooled RNA so there are no error margins. However this demonstrates the possibility of decidual-like changes in cervical tissue as has been shown in the endometrium.

EP2, EP4, PR, PDEIV and VEGF message was not significantly altered between groups. Laminin appears to be increased in the pregnant group, but this is due to a single greatly elevated result. There was a wide variation in data for all the results in the biopsy data.

Table 4.1 *RNA expression in untreated cervical biopsies; mean ± s.e.m (range)*

	Non pregnant n=6	**Pregnant** n=6	
PRL	**1.0**	**14.6**	**pooled RNA**
IGFBP-1	**1**	**52.8**	**pooled RNA**
PR	**1.1 ± 0.2** (0.6-2.6)	**2.3 ± 0.3** (1.6-4.1)	
EP2	**2.5 ± 0.9** (0.2-8.4)	**3.6 ± 1.7** (0.4-16)	
EP4	**3 ± 2** (0.1-13)	**5 ± 3.6** (0.1-23)	
PDEIV	**3.3 ± 1.9** (0.04-12.4)	**5.1 ± 3.1** (0.1-17)	
Laminin	**2.7 ± 1.1** (0.1-8.9)	**14.9 ± 9.1**(0.2-81)	
Fibronectin	**2.1 ± 0.6** (0.1-5.4)	**2.8 ± 1.3** (0.2-10.2)	
VEGF	**2.1 ± 1.3** (0.2-8.3)	**3.6 ± 2.7** (0.3-17)	

4.3.2 Cervical Stromal Cell response to decidual stimulus

a) Morphology

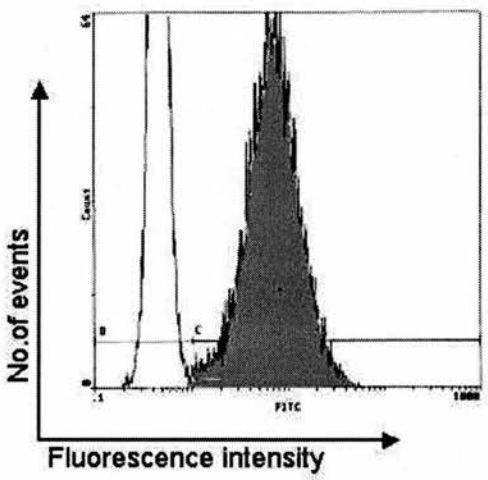
Spindle-shaped fibroblast-like cells grew out of cervical tissue from both pregnant and non-pregnant women. These will be referred to as cervical stromal cells (CSC) throughout the commentary. The fibroblast identity of these cells was confirmed by FACS analysis. The percentage of cells that possessed this marker was between 95 and 98 % of the total (Figure 4.1).

Cervical stromal cells treated with progestin and 8-bromo cAMP for six or ten days had a plumper morphology than untreated cells cultured in the same way (Figure 4.2). This represents a change characteristic of "decidual" transformation achieved in a relatively short period. Morphological decidual change in endometrial stromal cells exposed to progesterone alone *in vitro* can take 14 days or more to occur (Lane *et al* 1994) although local experience suggests a response in decidualisation markers within 48 hours as is shown in other studies (Gellersen *et al* 2003). Addition of cAMP is known to accelerate this response.

There was no observable difference between pregnant and non-pregnant cell lines and the light micrograph demonstrates the morphological change between control and DM regimes after 10 days in pregnant CSC.

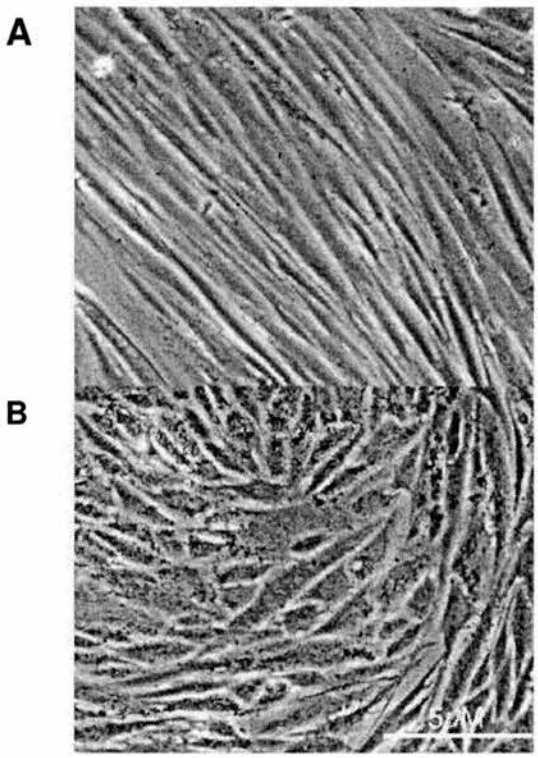
There was no difference in morphology between those CSC stimulated with DM (cAMP, MPA, oestradiol) or those with cAMP and MPA combined, without oestradiol.

Figure 4.1 *FACS Analysis CSC with fibroblast specific antibody (oncogene)*



FACS analysis of undifferentiated cultured human cervical stromal cells to confirm fibroblast nature.
Y axis = forward scatter
X axis = fluorescence intensity denoting specific fibroblast marker.
Cells positive for the fibroblast marker are shown in grey = 98% of cells

Figure 4.2 *Morphological change in pregnant CSC with decidual stimuli*



Cervical stromal cells grown for 10 days in (A) culture medium or (B) in the presence of 8-bromo-cAMP and MPA. Photomicrograph of cultured cell appearance after 10 days. Note cells treated with decidual stimuli are much more rounded in appearance.

b) Gene expression of decidualisation markers

These cervical stromal cells responded to stimulation, with cAMP and MPA, by expressing PRL and IGFBP-1 message (Figure 4.3). Both pregnant and non-pregnant CSC showed an increase in decidual marker gene expression, but the pregnant CSC response was much more marked.

Pregnant CSC (n=5) cultured with DM for six days effected a significant 26-fold (± 5 s.e.m, $p< 0.01$) increase in PRL mRNA expression, where cAMP alone produced a four-fold (± 0.8 s.e.m) increase above control.

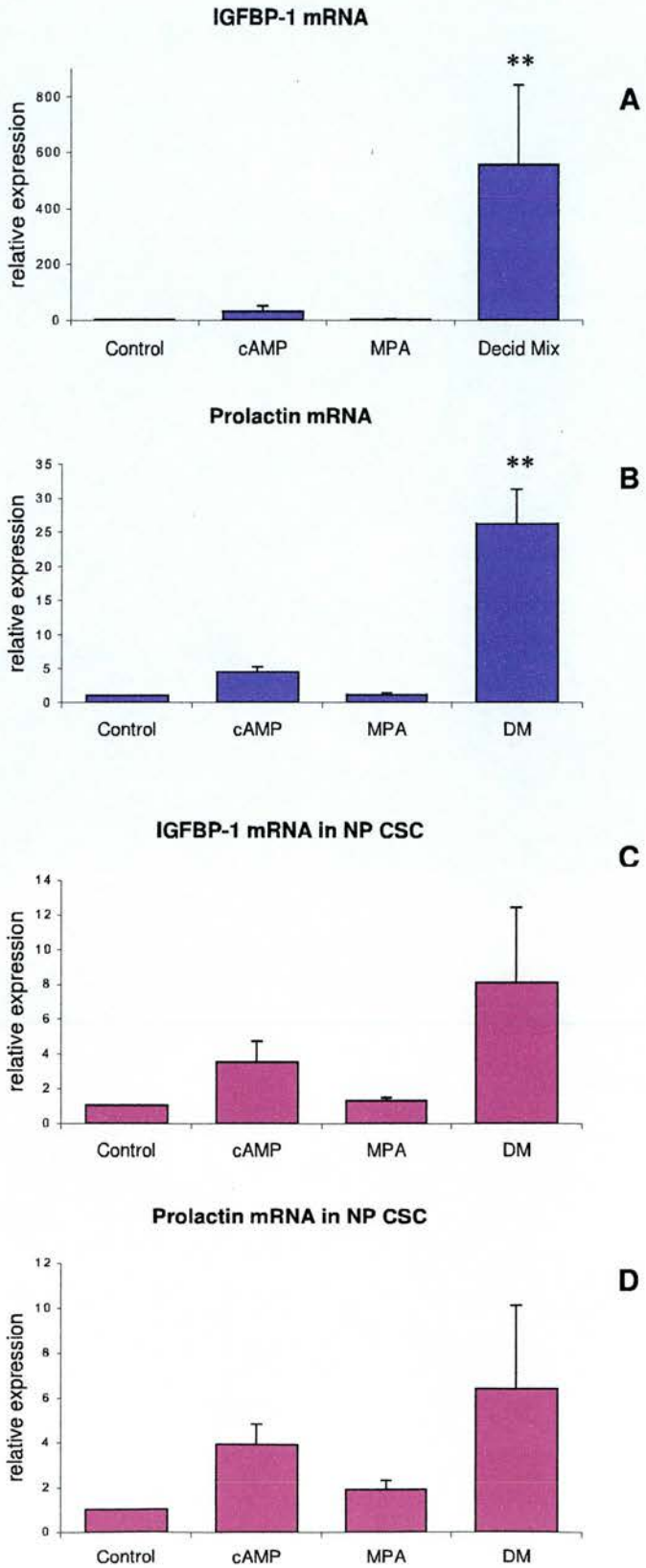
The same experiment with non-pregnant CSC gave a much more modest effect. The cAMP group resulted in a 3.9-fold increase (s.e.m ± 0.9) above control, compared with DM (6.4 ± 3.8; ns).

IGFBP-1 mRNA expression was increased 559-fold (s.e.m ± 282) and 8-fold (s.e.m ± 4.3) with DM, relative to control in pregnant and non-pregnant CSC respectively.

The effects of either progestin alone or cell-permeable cAMP analog (8-bromo-cAMP) alone were minor and not significant. However combined treatment produces significant effects consistent with a decidual-like response.

The addition of oestradiol to cAMP +MPA as a treatment regime did not alter the mRNA expression of the two major decidualisation markers in pregnant CSC (n=4), when compared to cAMP + MPA. IGFBP-1 mRNA expression was upregulated 148-fold (± 73 s.e.m) with cAMP and MPA, and was not significantly different with DM (including oestradiol) at 252-fold relative expression (± 85 s.e.m). PRL mRNA expression increased 12.6-fold (± 5.8 s.e.m) following cAMP + MPA exposure, compared to 16.1 ± 7.8 with the addition of oestradiol. See figure 4.4.

Figure 4.3 *Prolactin and IGFBP-1 mRNA expression in cervical stromal cells*



The increase in expression of IGFBP-1 and PRL mRNA is seen after 6 days differentiation with 8bromo-cAMP and MPA in combination.

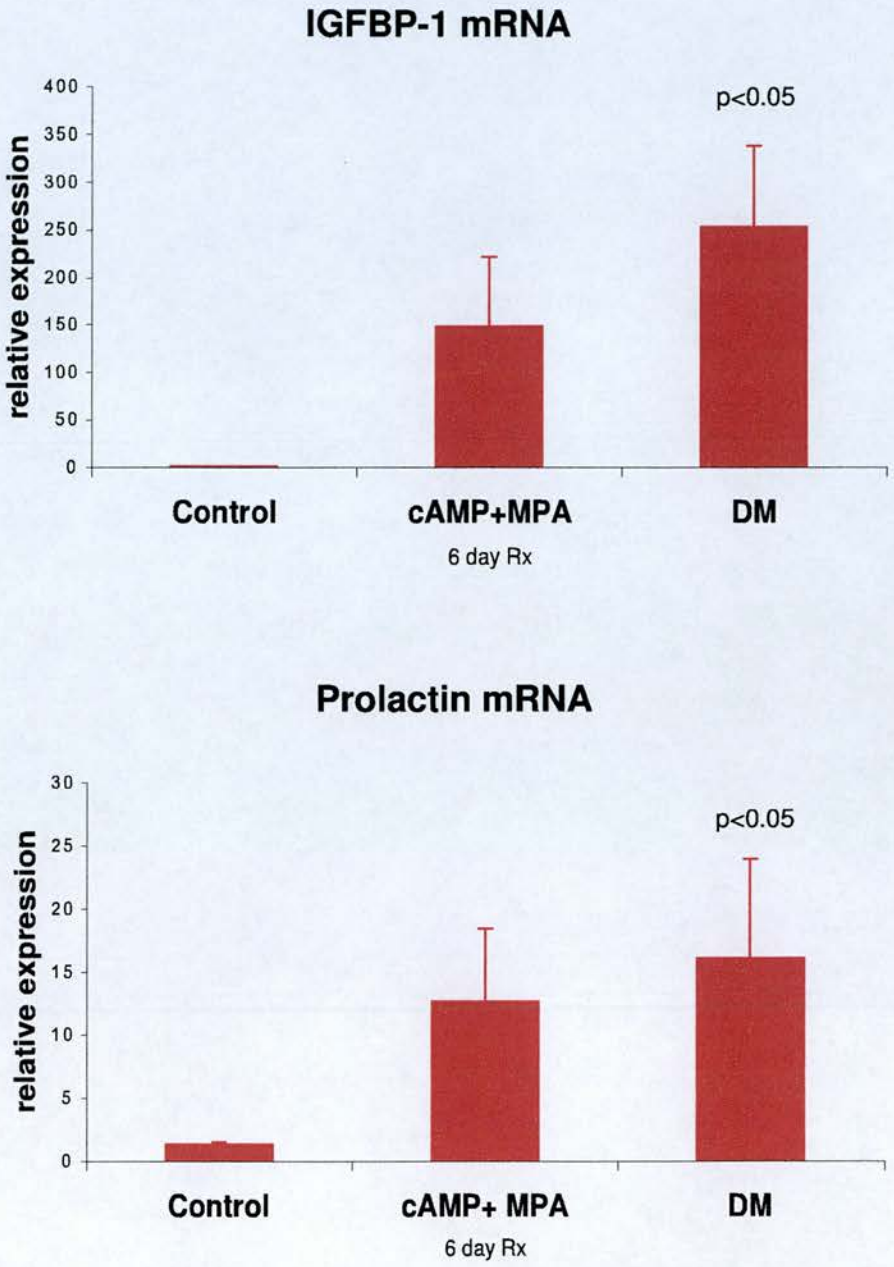
Graphs A and B demonstrate results from pregnancy-derived cells (n=5) and are expressed as mean – standard error of the mean.

Graphs C and D show the same for non-pregnancy-derived cells (n=6).

Although progestin alone is without effect, it is essential for the expression of decidualisation markers.

**p<0.01

Figure 4.4 *Prolactin and IGFBP-1 mRNA expression in pregnant CSC treated with DM ± oestradiol*



Comparison between cAMP+ MPA ± Oestradiol, where DM = cAMP + MPA + Oestradiol. Results shown as mean ± s.e.m. There is a significant ($p<0.05$) difference with DM relative to control, but here is no significant difference between the two treatment arms, therefore oestradiol has a negligible effect on the mRNA expression of the major decidualisation markers after 6 days.

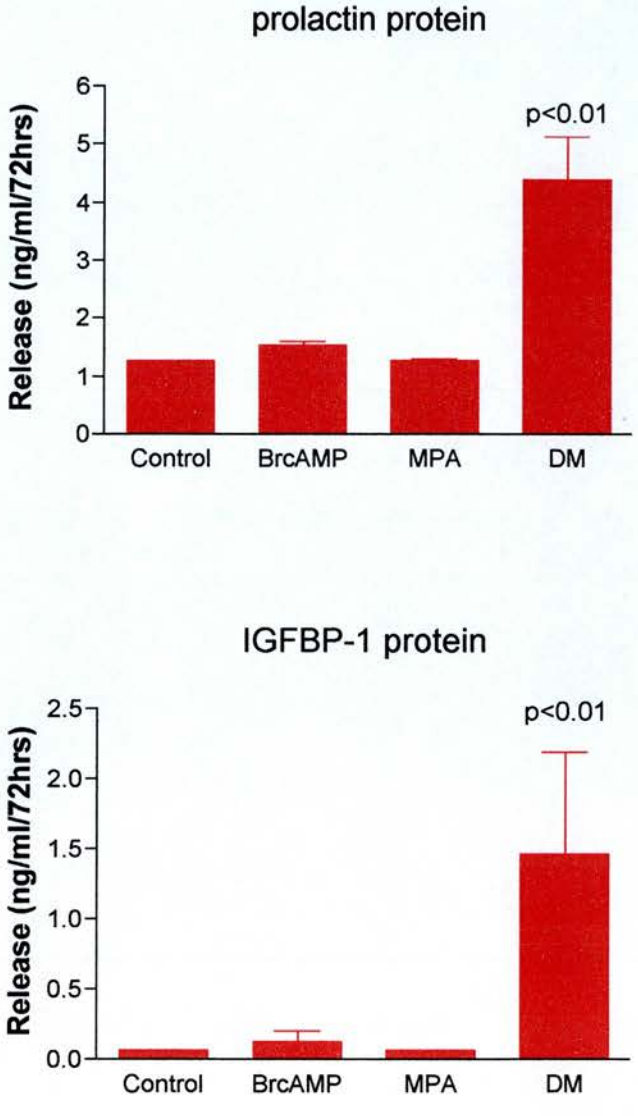
c) Protein release of decidualisation markers

Cervical stromal cells release more PRL and IGFBP-1 protein, following treatment with DM, than after treatment with cAMP or MPA alone. (Figure 4.5)

Pregnant CSC treated with DM produced 0.35ng/ml/72hrs (± 0.06 s.e.m.) PRL protein compared to control (0.1ng/ml/72hrs, $p<0.01$). Neither cAMP nor MPA alone had a significant effect, suggesting the combination is essential. Non-pregnant cells were not compared. See later for comparison of decidualisation regimes in both pregnant and non-pregnant CSC.

In pregnant CSC, IGFBP-1 protein release was significantly synergistically enhanced with DM (1.5ng/ml/72hrs ± 0.72) compared to control (0.06ng/ml/72hrs, $p<0.01$). Again, no effect is seen with either cAMP or MPA alone and there was negligible release in non-pregnant CSC in all treatment arms and control.

Figure 4.5 *Protein release of prolactin and IGFBP-1; pregnant CSC*



Prolactin and IGFBP-1 protein release, as measured by ELISA after a 6 day treatment period with cAMP, MPA, or DM. Results expressed as ng/ml/72hrs and shown relative to control as mean ± s.e.m. Significance is shown $p < 0.01$ relative to control.

d) **Receptor status**

The decidualised cells showed an increase in progesterone receptor mRNA expression in accord with observations in endometrium (Tseng *et al* 1997). Pregnant CSC (n=5) cultured with DM expressed a four-fold increase PR mRNA relative to control ($p< 0.05$) with no significant difference with cAMP or MPA treatment alone. (see Figure 4.6)

Non-pregnant CSC (n=6) showed no significant increase in PR mRNA with any treatment group compared to control (results not shown).

Accompanying changes in decidualisation, were increases in agents that might maintain a high intracellular cAMP such as the PGE_2 receptor type2 (EP2). Again pregnant CSC (n=5) responded synergistically when exposed to both cAMP and MPA with a 5.6-fold increase in EP2 receptor mRNA expression, $p< 0.05$. (see figure 4.6)

Comparatively, non-pregnant CSC (n=6) under the same conditions did not show the same synergistic response with slightly raised EP2 mRNA expression (2 to 3-fold) in all treatment groups. There was no significant difference between groups with wide variation within groups.

EP4 expression did not vary following 6 days treatment with cAMP, MPA or DM (control 1.01 ± 0.01; DM 0.77 ± 0.33) in pregnant CSC.

Similarly, the previously demonstrated rise in PR and EP2 receptor expression was consistent with DM or cAMP + MPA stimulation alone, suggesting no significant oestradiol effect. See Figure 4.7.

Figure 4.6 *Receptor mRNA expression in pregnant CSC (mean ± s.e.m)*

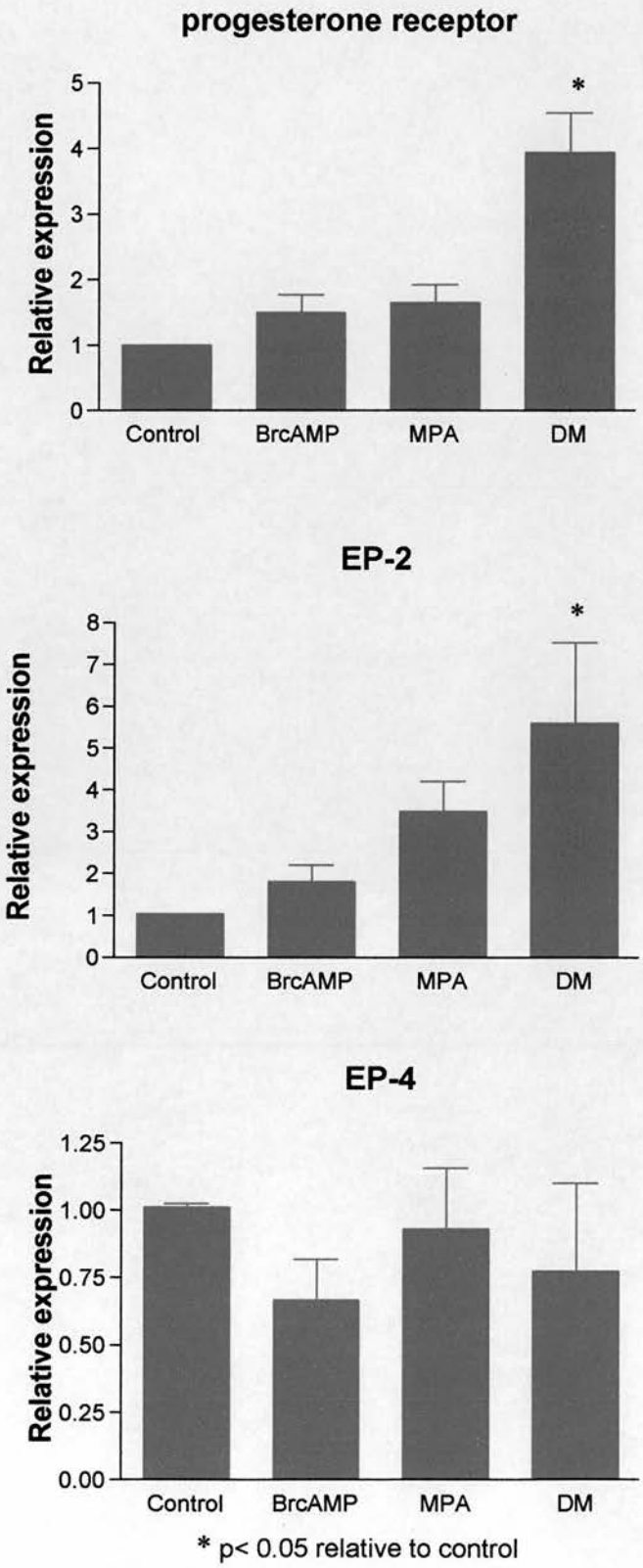
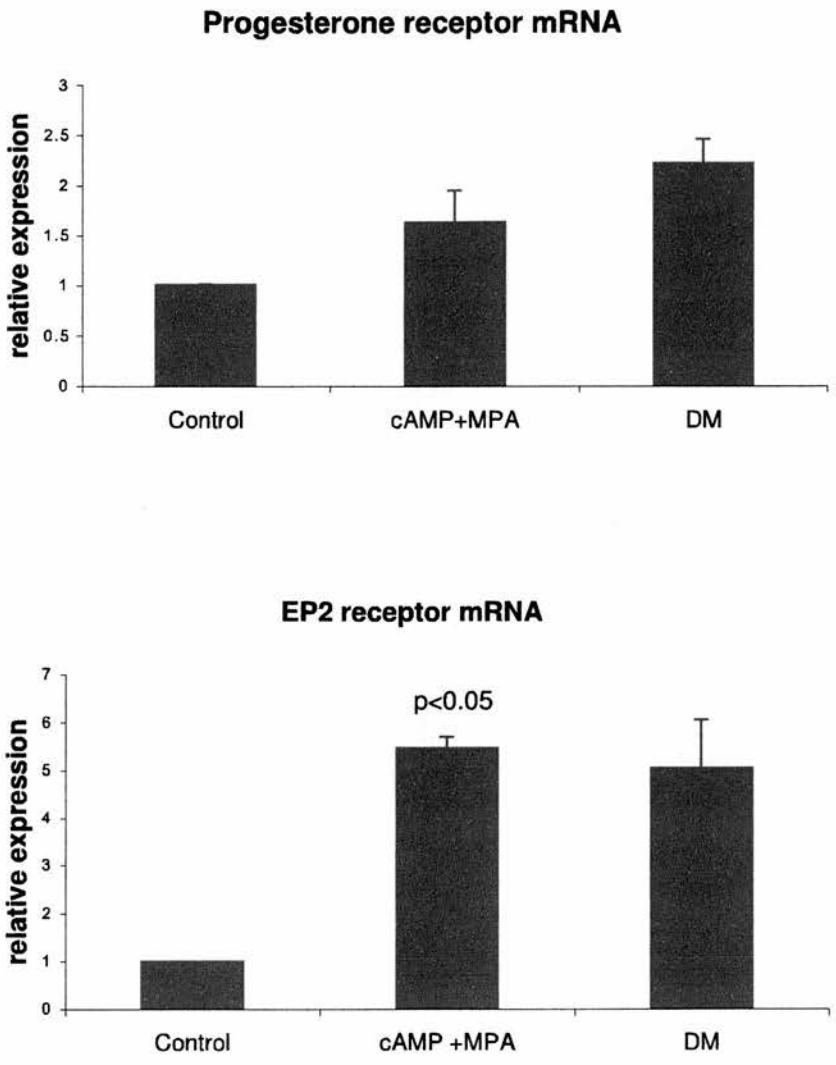


Figure 4.7 *Decidual stimuli (cAMP + MPA) ± oestradiol in pregnant CSC, n=4*



Both PR and EP2 are upregulated in response to cAMP + MPA (PR 1.63 ± 0.31; EP2 5.46 ± 0.23), but this is only significant for EP2 ($p<0.05$) relative to control. The addition of oestradiol had no additional significant effect (PR 2.2 ± 0.24; EP2 5.05 ± 1.01) in either group. Results are expressed as mean ± s.e.m.

e) Surface marker expression

The CD10 surface marker, as determined by FACS analysis, was found to be present in both pregnant and non-pregnant CSC as controls. Following identical culture conditions for 10 days a variation in CD10 expression was seen after DM treatment depending on origin of CSC.

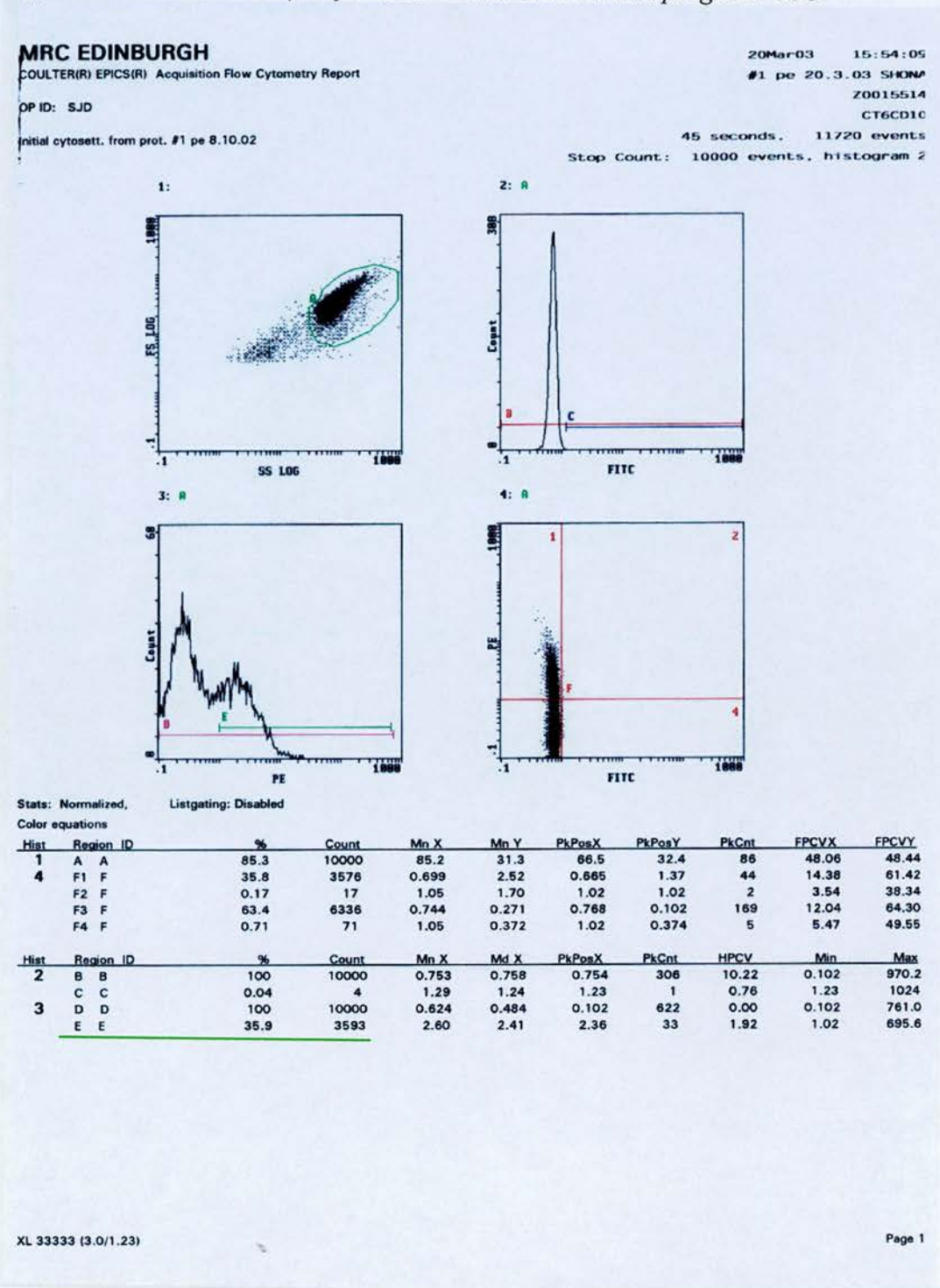
In non-pregnant CSC control CD10 expression was 42% (as mean of two cell lines, st.dev 5.4%) whereas CSC treated with DM for 10 days resulted in a decreased CD10 expression of 15.5% (st.dev 1.8%).

Alternatively, in pregnant CSC control CD10 expression was 25.9% (as a mean of two cell lines, st.dev 14.1%) and increased non-significantly to 35.2% (st.dev 20.3%) following DM stimulation.

Examples of the FACS results are shown in Figure 4.8 and 4.9. This represents the proportion of each cell population positive for the CD10 marker. The example shown is a pregnancy-derived cell line cultured as control in Figure 4.8 and following 10 day DM treatment in Figure 4.9.

Results of both pregnancy and non-pregnancy-derived cells are summarised in Figure 4.10.

Figure 4.8 *FACS Analysis for CD10 in cultured control pregnant CSC*

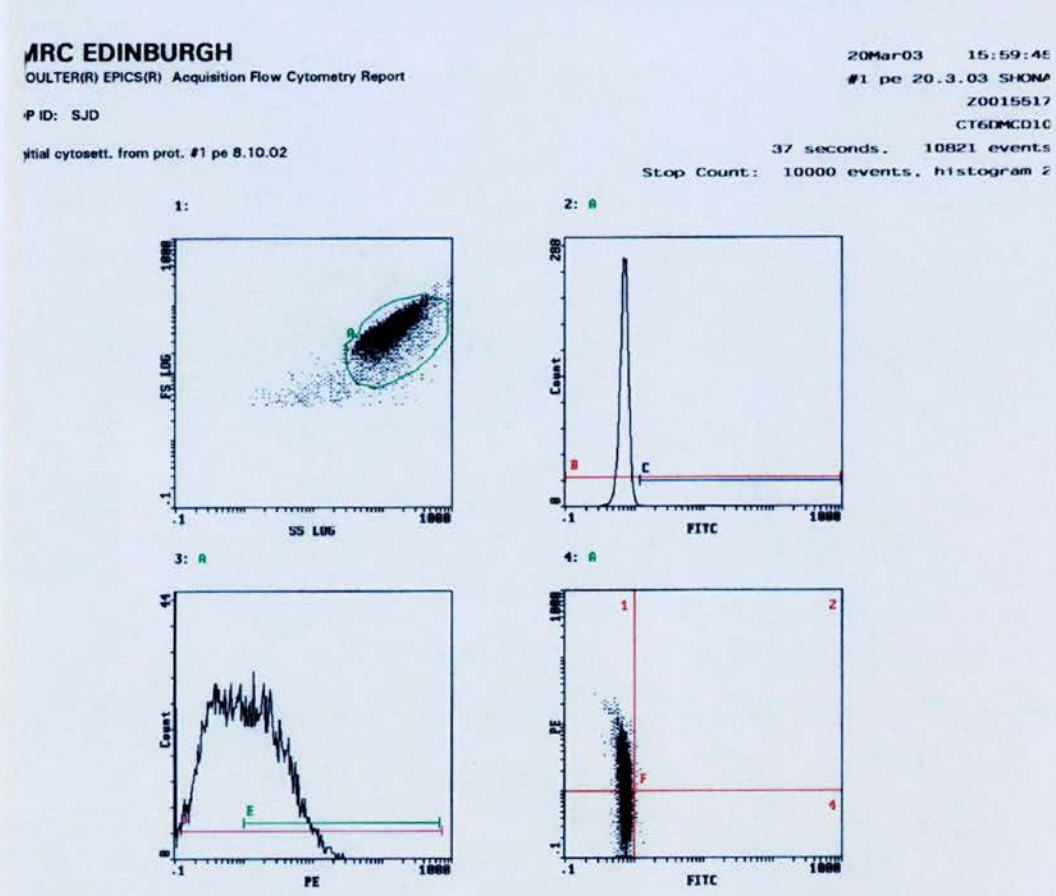


Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	85.3	10000	85.2	31.3	66.5	32.4	86	48.06	48.44
4	F1	F	35.8	3576	0.699	2.52	0.665	1.37	44	14.38	61.42
	F2	F	0.17	17	1.05	1.70	1.02	1.02	2	3.54	38.34
	F3	F	63.4	6336	0.744	0.271	0.768	0.102	169	12.04	64.30
	F4	F	0.71	71	1.05	0.372	1.02	0.374	5	5.47	49.55

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	100	10000	0.753	0.758	0.754	306	10.22	0.102	970.2
	C	C	0.04	4	1.29	1.24	1.23	1	0.76	1.23	1024
3	D	D	100	10000	0.624	0.484	0.102	622	0.00	0.102	761.0
	E	E	35.9	3593	2.60	2.41	2.36	33	1.92	1.02	695.6

The CD10 positive (phycoerythrin-labelled) cells are represented by the green line E in histogram 3. The same data is underlined in green in the lower table, as a percentage of the cell population. These results represent pregnancy-derived CSC in control culture conditions for 10 days prior to FACS.

Figure 4.9 *Pregnant CSC exposed to decidual stimuli – FACS for CD10*

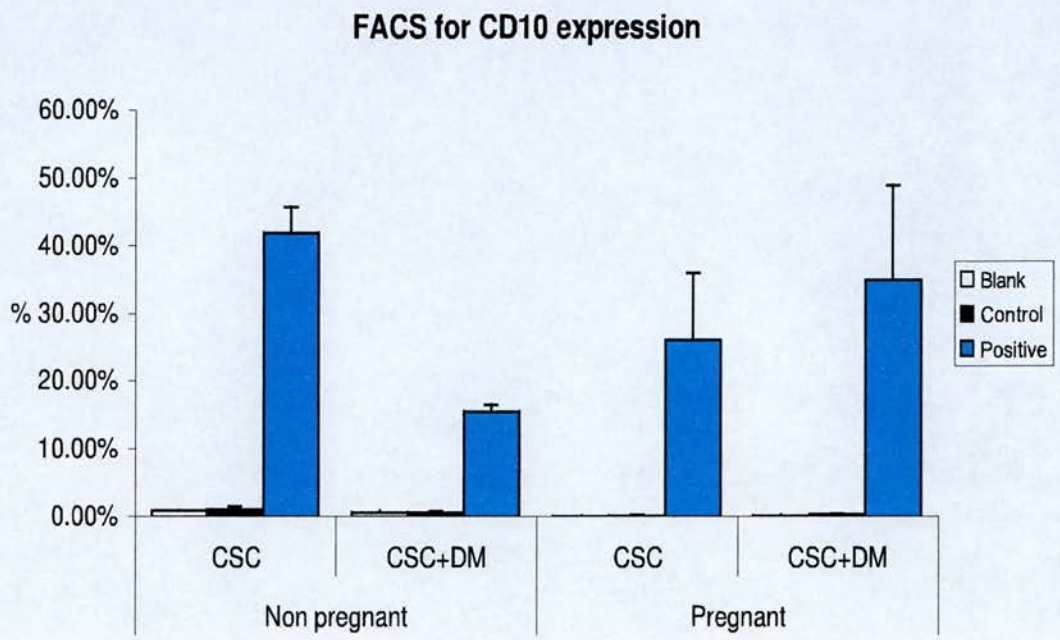


Hist	Region	ID	%	Count	Mn X	Mn Y	PkPosX	PkPosY	PkCnt	FPCVX	FPCVY
1	A	A	92.4	10000	132.1	38.4	102.4	46.4	50	64.07	57.70
4	F1	F	49.1	4910	0.687	2.71	0.768	1.10	59	15.85	69.12
	F2	F	0.38	38	1.08	2.21	1.02	1.27	2	7.95	61.40
	F3	F	49.9	4989	0.739	0.383	0.768	0.432	56	12.28	61.52
	F4	F	0.63	63	1.06	0.486	1.02	0.402	4	7.35	46.04

Hist	Region	ID	%	Count	Mn X	Md X	PkPosX	PkCnt	HPCV	Min	Max
2	B	B	100	10000	0.738	0.745	0.768	287	11.61	0.102	970.2
	C	C	0.09	9	1.35	1.33	1.26	1	1.53	1.23	1024
3	D	D	100	10000	1.04	1.00	0.102	184	0.00	0.102	761.0
	E	E	49.5	4948	2.79	2.53	1.44	38	4.47	1.02	695.6

The CD10 positive (phycoerythrin-labelled) cells are represented by the green line E in histogram 3. The same data is underlined in green in the lower table, as a percentage of the cell population. These results represent pregnancy-derived CSC in DM culture conditions for 10 days prior to FACS.

Figure 4.10 *FACS Analysis of CD10 expression in cervical stromal cells*



Graph shows FACS analysis for CD10 in both pregnant (n=2) and non-pregnant (n=2) cell lines. The above applies to cultured CSC after 10 day treatment with control or DM medium. White bars represent the blanks, black bars the isotype controls and blue bars the proportion of the cell population positive for CD10. Decidualisation stimuli produced a reduction in CD10 expression in non-pregnancy-derived cell lines, whereas the opposite was seen in pregnancy-derived cells. Results are expressed as mean – s.e.m.

CD 10 expression in the control non-pregnant cell lines (n=2) was mean 42%(range38-46%), and decidual stimulus resulted in a significant reduction to mean of 15.5% (range14-17%). Significance; unpaired Mann-Whitney U two-tailed p value <0.0000.

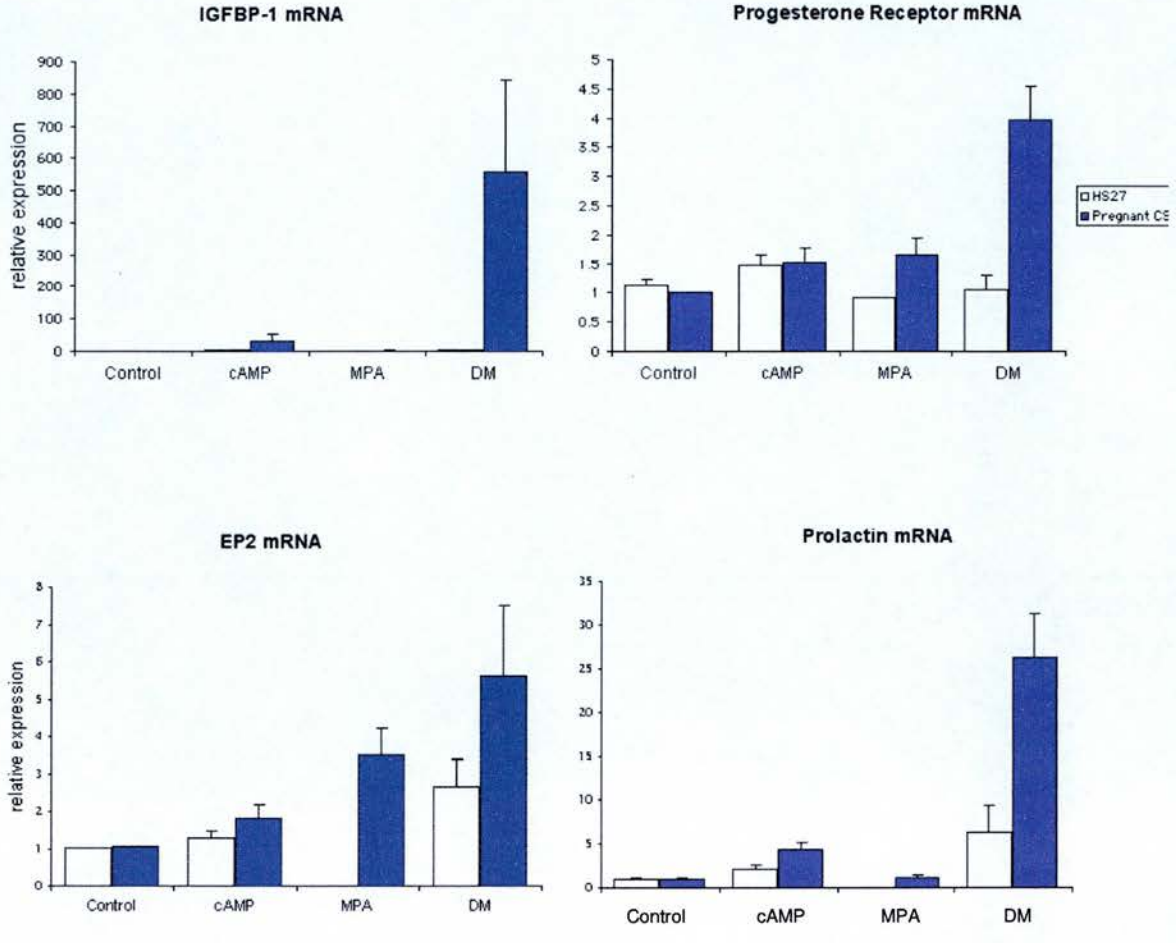
In the pregnancy-derived cell lines (n=2) CD 10 expression was a mean of 26% (range16-36%) in the control group compared to 35% (range 21-50%) in the treated group (ns).

4.3.3 Comparison to non-reproductive cell line

HS27 fibroblasts did not produce the same response to decidual stimulus in identical culture and treatment conditions.

The response of the HS27 cells to DM incubation was a reduction in IGFBP-1 message and no change in PRL message. There was no increase in PR or EP2 receptor expression in the foreskin cell line on exposure to decidual stimuli, as shown with the cervical stromal cells. (See Figure 4.11)

Figure 4.11 *Comparison of pregnant CSC with HS27 (foreskin fibroblast cell line)*



Identical culture and treatment regimes were used for both pregnant CSC and HS27 (foreskin fibroblast) cell lines. The purple bars represent pregnancy-derived CSC and the white bars represent HS27 cells.

Previously shown significant increases in decidualisation markers, PRL and IGFBP-1 mRNA expression in CSC, are not duplicated in HS27. Similarly PR and EP2 receptor mRNA expression did not vary in treatment arms with HS27. This confirms a response particular to a reproductive fibroblast cell line.

4.4 DISCUSSION

Changes in the consistency of the cervix can be detected in early pregnancy (Uldbjerg *et al* 1990) but little is known about the contribution of the various cell types within the cervix to such a change. The stromal cell of the cervix has been considered an important contributor to physiological changes, both in early pregnancy and at the time of parturition. These cells respond to steroids and initiate collagenolytic pathways (Sato *et al* 1991; Ito *et al* 1994; Carbonne *et al* 2000; Schmitz *et al* 2001) and since they have progesterone receptors, they may play a sentinel role in altering cervical morphology in response to hormone changes. However if these cells respond to prolonged exposure to progesterone with a change in phenotype, as do cells in the endometrium, then this modified cell may be critical in the maintenance of normal cervical function in pregnancy. In support of this hypothesis I have shown that human cervical stromal cells have the capacity for a phenotypic alteration akin to decidualisation in pregnancy and that this can be induced *in-vitro* by a combination of progesterone and cAMP.

The cervical biopsy data suggests an increase in both PRL and IGFBP-1 mRNA expression in first trimester cervical tissue relative to non-pregnant cervical tissue. As has been shown, the wide variation seen with biopsy samples can make analysis difficult. This could be for many reasons including age, parity, gestation or stage in menstrual cycle. The limited tissue available limits the number of investigations one can perform. In addition, although the biopsies were all taken in an identical fashion, it is impossible to ensure equal contribution from differing cell types. For these reasons pooled RNA was used to evaluate the presence of a trend for further exploration. To investigate this further with particular emphasis on the cervical fibroblast, cervical stromal cell cultures were established for *in vitro* studies of the response of these cells to decidual stimuli.

These primary cells were identified as fibroblasts by a fibroblast specific antibody. Another method of fibroblast identification is by immunocytochemical staining for

vimentin, a typical marker for fibroblasts or mesenchymal cells which is absent on epithelial cells (Brar *et al* 2001). Other investigators have demonstrated 99% vimentin positivity in cultured human cervical fibroblasts after similar culture conditions and use of passage numbers, with <1% of these cells possessing α-smooth muscle actin (Yoshida *et al* 2001; Yoshida *et al* 2002; Takemura *et al* 2004).

I show here that both pregnancy and non-pregnancy-derived cervical stromal cells respond to progestins in the presence of cAMP by undergoing "decidual" changes, as seen in endometrial stromal cells. Morphologically this is primarily represented by a change in cell shape to a more rounded form. Both of the major decidualisation markers, PRL and IGFBP-1, are up-regulated significantly with a combination of cAMP and MPA. Oestradiol does not appear to influence these changes. This has been shown by both mRNA expression and protein release and is particularly marked in the CSC derived from pregnant women. Perhaps this is due to pregnancy associated phenotypic alteration *in vivo* prior to culture conditions. In support of these changes we also see increased PR and EP2 receptor mRNA expression (Tseng *et al* 1997).

Of significance is the finding that fibroblasts derived from foreskin do not produce the same response. There is mild prolactin stimulation but no IGFBP-1 effect as has been reported in skin fibroblasts (Richards *et al* 1996).

Although progesterone appears to be sufficient for the decidual change *in vivo*, cells *in vitro* need to be sensitised by elevated intracellular cAMP levels (Brosens *et al* 1999). These findings confirm the same to be true for cervical stromal cells. *In vivo*, raised intracellular cAMP levels can be brought about by prostaglandin E acting through either EP2 or EP4 receptors to activate the stimulatory G-protein (Gs) or by relaxin inhibiting the enzyme responsible for the catabolism of cAMP, phosphodiesterase (Bartsch *et al* 2001). Since one effect of raising cAMP is to stimulate expression of phosphodiesterase both of the above pathways will work together in a synergistic fashion to maximise cAMP levels. Other agents involved in decidualisation may also affect phosphodiesterase activity since epidermal growth

factor inhibits the activity of an isoform of PDEIV by ERK-2 dependent phosphorylation (Hoffmann *et al* 1999).

The cervical biopsy data supports the presence of up-regulated PRL, IGFBP-1 mRNA expression in first trimester cervical tissue, but clearly this is not comparative as these results are from whole tissue biopsies and are therefore widely variable and include other cell types and possible interactions. The wide variation seen in biopsy RT-PCR results for laminin, fibronectin and VEGF as other markers of decidualisation should also be assessed in the controlled CSC model, or by immunolocalisation.

The CD10 surface marker reported in decidual stromal cells (Montes *et al* 1996; Oliver *et al* 1999; Kimatrai *et al* 2003) did not show consistent changes in pregnancy and non-pregnancy derived cells, with a trend to up-regulation in CD10 expression in pregnant CSC after decidual stimulus, but a down regulation in non-pregnant CSC. This effect may be due to length of exposure to decidual stimulus, in that the pregnancy-derived tissue will have been exposed physiologically for several weeks before culturing without decidual stimuli. This is then followed by re-exposure *in-vitro* where a temporarily interrupted phenotypic alteration may re-establish. It is possible that the duration of exposure required for CD10 expression as a decidual marker is longer than 6 or 10 days in those non-pregnant CSC not previously exposed. The surface-expressed zinc dependent metalloproteinases, CD10 and CD13, were expressed on 95% of cultured preDSC whereas no group of cervical cells studied here reached that degree of expression. Perhaps the origin of cervical and endometrial stromal cells are different, or perhaps the cyclical local environment of ESCs predisposes a heightened response.

It would be of paramount importance to confirm these findings in-vivo and immunohistochemical confirmation in cervical biopsies would be ideal. Localisation studies would provide information on the functional contribution of differing cell types and it would be of particular interest to explore varying gestational stages. Detailed localisation of these "decidualised" fibroblasts may reveal a spatial

relationship with the blood vessels as has been reported in the endometrium (Kelly *et al* 2002). The exact purpose of decidualisation in the endometrium is still a mystery but angiogenic changes are clearly important and may also apply to the cervix.

Although decidualisation of the cervical fibroblast has not hitherto been recognised, ectopic decidua has been reported on cervix and ovary during pregnancy (Zaytsev *et al* 1987; Massi *et al* 1995). There has always been doubt whether this was a manifestation of otherwise unrecognised endometriosis. The identification of the decidual marker IGFBP-1 immunolocalised to decidual-like cells of extrauterine sites, including the cervix, supports the potential demonstrated in this *in vitro* model (Rutanen *et al* 1991).

The cervical fibroblast has the potential to orchestrate tissue remodelling in all stages of pregnancy, or even in menstruation. What is not clear is the function of these phenotypically altered cells. Gene expression and protein secretion resemble endometrial DSC, yet the function of this endometrial transformation is unknown. It is essential for successful implantation and trophoblast invasion, and appears to involve MMP modulation, angiogenesis and vaso-reaction. Menstruation occurs with progesterone withdrawal once endometrial stromal cells have undergone decidualisation (Lockwood *et al* 1998; Warren *et al* 1999). It is possible that decidualised cervical stromal cells of pregnancy are therefore primed to respond to a localised functional progesterone withdrawal towards term, with the effect of cervical ripening. The mechanism of local progesterone functional withdrawal in human parturition is unclear but the concept is favoured as a necessary step (Allport *et al* 2001; Mesiano *et al* 2002). This raises the possibility of altered function of these cervical cells at differing stages of pregnancy. The implications of this vary from providing cervical integrity for the majority of pregnancy to a change in status resulting in cervical ripening, whether this occurs physiologically at term, preterm or in response to infection. Not only are the characteristics of this altered cell-type important, but the influence on surrounding cells, blood vessels and extracellular matrix. Understanding the interaction between other resident and migratory cells will be paramount in appreciating the role of the "decidualised" cervical fibroblast.

These functions may include recruitment and activation of leukocytes, paracrine communication between cells and cAMP modulators or initiation of the matrix remodelling cascade.

These findings on the decidual-like changes in cervical stromal cells open a new area for the study cervical function throughout pregnancy. Any advances in the knowledge of physiological cervical changes in pregnancy can be exploited in attempts to manipulate events in preterm labour or prolonged pregnancy.

CHAPTER 5

CHARACTERISATION OF THE "DECIDUALISED" CERVICAL STROMAL CELL

5.1 INTRODUCTION

The ability of cervical stromal cells to undergo a decidual-like reaction in the same manner as endometrial stromal cells suggests that these altered cells may have a specific role in cervical function in pregnancy. The physiological mechanism of decidual stimulation may be complex but progesterone is critical and varied mechanisms resulting in cAMP elevation are certainly effective *in vitro* and present during pregnancy (Gellersen *et al* 2003).

Implantation is associated with significant vascular changes and leukocyte influx (King *et al* 2000). Another marker of endometrial decidualisation is tissue factor, a membrane-bound inhibitor of bleeding, thought to control haemorrhage at the time of vascular remodelling as the trophoblast invades the maternal spiral arteries (Lockwood *et al* 1993). Localised angiogenesis may be regulated by TF-enhanced autocrine VEGF stimulation (Lockwood *et al* 2002). Clearly, cervical trophoblast invasion would not occur in normal circumstances, but structural and vascular changes do occur even early in gestation and VEGF may be partially responsible (Sugino *et al* 2002). Increased vascular permeability may promote controlled leukocyte infiltration. Progesterone levels are thought to restrict excessive neutrophil entry and this has been well described in a sheep model (Staples *et al* 1983). Progesterone antagonism increases PGE_2, chemotactic agents and leukocyte numbers. PR present on DSC are therefore key in this role. VEGF may be partly responsible for the vascular permeability changes in the pregnant cervix, as in the decidualisation reaction, as expression of VEGF and its receptors is up-regulated in human decidual cells (Sugino *et al* 2002).

Fibronectin, laminin, β2-microglobulin and desmin have been described as potential markers of endometrial decidualisation, and alteration of these components can be seen in tissue remodelling (Aplin *et al* 1988; Loke *et al* 1989; Komatsu *et al* 1998). Evaluation of cervical biopsy whole tissue in the first trimester did not demonstrate a similar induction in mRNA, but there was wide variation in these samples. Further

assessment of these markers will be undertaken in purified cervical stromal cell culture conditions.

Myofibroblastic characteristics have been identified in human DSC and in the human cervix at term (Oliver *et al* 1999; Montes *et al* 2002; Kimatrai *et al* 2003). These include the presence of cytoskeletal filaments desmin and α-smooth muscle actin and evidence of contractility or ultrastructural features consistent with the phenotype. The presence of desmin and smooth muscle actin strongly suggests a contractile function, inhibited by progesterone and activated by cytokines (Kimatrai *et al* 2003). Rat cervical fibroblasts display high cytoskeletal desmin intensity but not until late in pregnancy in association with ultrastructural appearances of secretory myofibroblasts (Varayoud *et al* 2001).

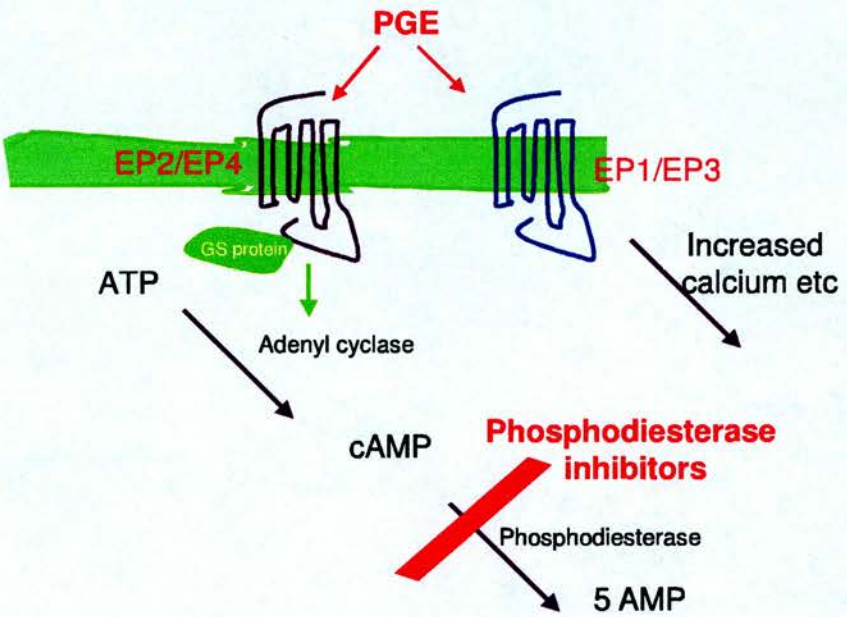
Cervical changes in labour are dramatic and associated with pro-inflammatory changes, in particular leukocyte infiltration and cytokine upregulation. IL-8 has been shown to be induced in cervical tissues in labour (Barclay *et al* 1993; Winkler *et al* 1998; Winkler *et al* 1999b; Sakamoto *et al* 2004) and has been used in animal models to produce cervical ripening as has been discussed in Chapter 1 (Chwalisz *et al* 1994; El Maradny *et al* 1994; 1996). It is postulated that there is a synergism between PGE and IL-8 in the mechanism of cervical ripening, with a combined effect of vaso-reaction and chemotaxis. In concert, this activates an inflammatory cascade, triggering ECM remodelling via proteoglycan reorganisation and enzyme degradation of collagen and matrix proteins.

If IL-8 is a significant player in cervical ripening at term, then an evaluation of its production by cervical stromal cells exposed to pro-decidual influences is of relevance. Other investigators have established a recognised up-regulation of cervical IL-8 production in late pregnancy and labour (Winkler *et al* 1999b; Sakamoto *et al* 2004; Tornblom *et al* 2005). It is pertinent to assess the relative IL-8 expression in non-pregnant and first trimester cervical tissue and then assess the response with the CSC model and decidual stimulus.

Many *in vitro* models use 8-bromo-cAMP, an analogue resistant to PDE with increased potency. Alternative mechanisms for the physiological induction of decidual differentiation are likely. Cyclic AMP is required in *in-vitro* studies to effect decidualisation changes and progesterone is essential. There is potential convergence of the progesterone and prostaglandin pathways during stromal transformation, at the level of the EP2 receptor, stimulated by both PGE_2 and progesterone, effecting raised cAMP levels. Intracellular cAMP plays a central role in decidualisation via PKA signalling. Physiological cAMP stimulating agents include PGE_2, relaxin and phosphodiesterase inhibitors. EP2 is known to be the dominant PGE receptor in pregnant baboon cervices (Smith *et al* 1998). Cyclic AMP catabolism is mediated by phosphodiesterase, which is up-regulated with raised cAMP levels. Inhibitors of this enzyme (relaxin, rolipram) will prevent catabolism and thereby maintain raised cAMP levels (see Figure 5.1). Studies have shown that PDE inhibitors such as rolipram have a profound effect on decidualisation (Bartsch *et al* 2004). A combination of PGE_2, acting via cervical EP2 receptors, and rolipram should therefore mimic the cAMP effect, and in combination with progesterone, cause decidualisation.

Other factors involved in PGE synthesis and catabolism such as the COX enzymes and PDGH are relevant in physiological cAMP regulation. It must also be remembered that PGE_2 catabolism by PGDH is also progesterone dependent (Cheng *et al* 1993). PGE_2 and relaxin have been used to elicit cervical ripening (MacLennan *et al* 1986; Calder *et al* 1993; el-Refaey *et al* 1994; Brennand *et al* 1997; Stjernholm *et al* 1999; Witter 2000), and have been associated with endometrial decidualisation (Huang *et al* 1987; Lane *et al* 1994; Bartsch *et al* 2001). The effects of PGE_2 on the cervix will be dependent EP2 expression, phosphodiesterase activity and on progesterone status, mediated by PR expression.

Figure 5.1 *cAMP/prostaglandin pathway*



It is hypothesized that interaction between these pathways provides a physiological decidual stimulus via cAMP up-regulation in association with a progesterone rich environment. The aims of this section are to explore the potential consequences of the decidual-like changes seen in CSC by assessing phenotypic markers and relate this to mechanisms of cervical change in pregnancy.

Hypotheses:

1. Decidualised CSC will express decidual markers tissue factor and VEGF and demonstrate matrix protein induction as seen in the endometrium.
2. Cervical "decidualisation" down-regulates stromal IL-8 in early gestation providing a mechanism for IL-8 stimulation in labour when functional progesterone withdrawal occurs.
3. Cyclic AMP-elevating factors provide a physiological basis for decidual stimulus resulting in cervical stromal cell decidual-like reaction.

Aims:

1. Evaluate markers of decidualisation in CSC and compare to cervical biopsy findings, by RT-PCR.
2. Assess the response of CSC to decidual stimuli with respect to IL-8 expression and protein release.
3. To apply an alternative decidualisation regime (PRM) providing indirect cAMP stimulation, and compare response with DM. (PRM - PGE_2, rolipram and MPA)

5.2 METHODS

Tissue collection, culture, RNA extraction and RT-PCR processes were as described in Chapter 4.

Untreated cervical biopsy samples were homogenised and RNA extraction performed as before. Pregnancy (first trimester) and non-pregnancy-derived samples were compared by Q RT-PCR for gene expression of decidualisation markers, prostaglandin metabolism enzymes and IL-8.

For cell culture experiments, all cells were plated at 5 x 10^4/ ml complete medium in 6 well plates and grown to confluence over 72hrs before treatment commenced. Following a PBS wash treatments were prepared with 2% FCS supplemented RPMI (with 20µg/ml gentamicin, 100IU/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine).

Treatment regimes

RNA extracted from treatments groups in Chapter 4 was used to perform RT-PCR for other markers of decidualisation and matrix modulators:

1. Control
2. 8-bromo-cAMP 250µM (cAMP)
3. Medroxyprogesterone acetate 10^{-6}M (MPA)
4. Decidualising Mix (DM)

To explore the potential physiological process of prostaglandin and progesterone interaction an alternative decidual stimulus treatment was with PRM; PGE_2 (10^{-6}M), Rolipram (10^{-6}M) and MPA (10^{-6}M). For this comparison both pregnant cell lines (n=6) and non-pregnant cell lines (n=5) were used.

1. Control
2. DM
3. PRM

Treatment periods were for 6 or 10 days, with medium changes every 3 or 4 days. Medium was collected in duplicate at the end of each treatment period and stored at -20°C for protein assay at a later date as previously described.

Quantitative Real Time-PCR

RNA extracted from cervical biopsies as in Chapter 4, was used for Q RT-PCR to assess mRNA expression of IL-8, COX-1, PGDH.

Following in-vitro studies with decidual stimuli in Chapter 4, all medium was removed, RNA extraction and Q RT-PCR was performed as previously described.
To examine the gene expression of other known decidualisation markers in cultured CSC, RT-PCR was carried out for tissue factor (TF), desmin, laminin, fibronectin and β_2-microglobulin, phosphodiesterase type 4 (PDEIV), COX-1, COX-2, IL-8 and vascular endothelial growth factor (VEGF) in control and treatment groups.

Experiments were repeated under identical conditions with the alternative proposed decidual stimulus, PRM. Medium was stored and RNA extracted prior to Q RT-PCR for PRL, IGFBP-1, PR, fibronectin, laminin, and IL-8.

Enzyme Linked Immunosorbent Assays

Protein release estimation was performed in duplicate for all samples as previously described.

Prolactin protein release was measured using a PRL kit as described in Chapter 4.

The IGFBP-1 assay was performed as described in Chapter 2 with a lower limit of sensitivity of 62.4pg/ml and a within assay variation of 1.7% (relative standard deviation). Again, all samples were analysed on the same assay.

IL-8 protein release was measured by ELISA, with matched pairs of capture and biotinylated-detection antibodies for IL-8 as described in Chapter 2.

5.3 RESULTS

5.3.1 Gene expression of decidualisation markers in treated CSC

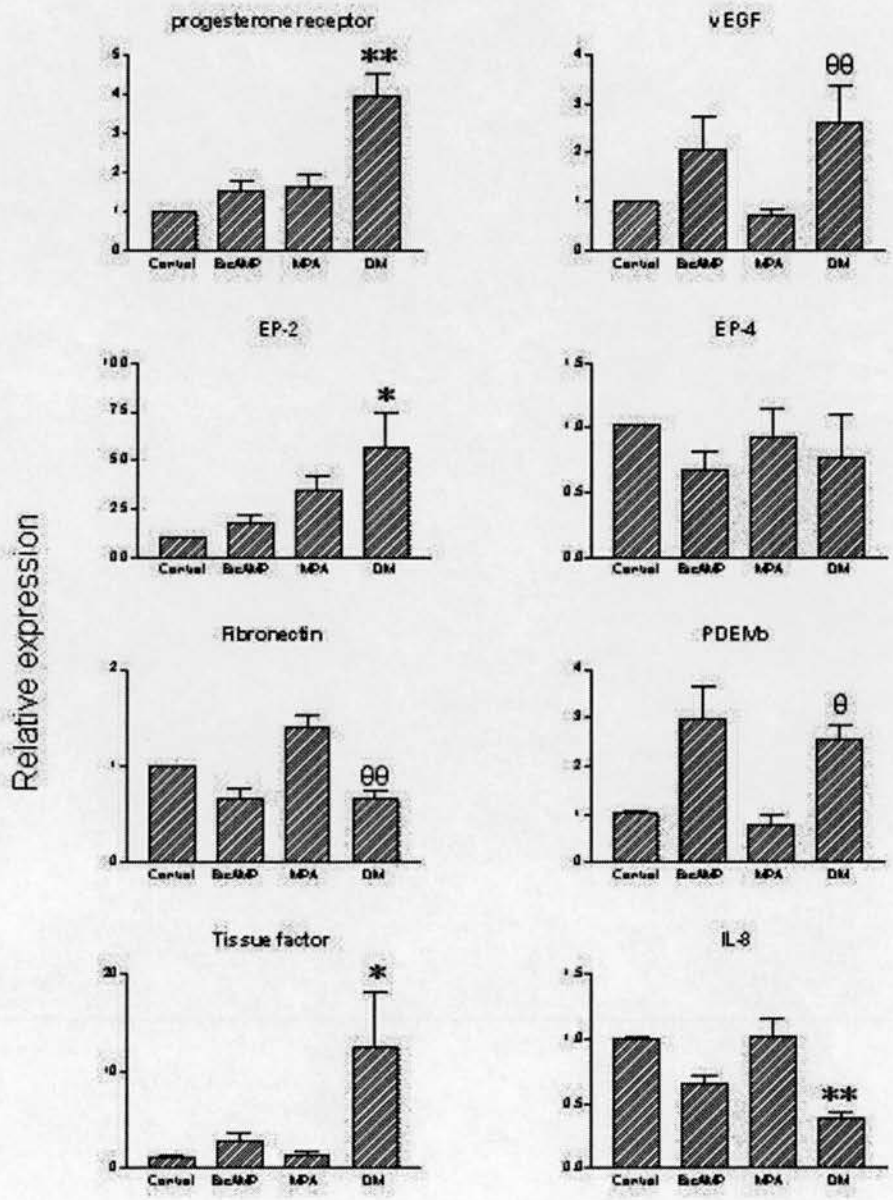
The following results relate to pregnant CSC unless otherwise stated. Treatment arms are either control, cAMP, MPA or DM as described in Chapter 4.

Tissue factor, a further decidualisation marker, mRNA expression is significantly increased with DM treatment relative to control (mean 12.6 ± 5.6 s.e.m; $p<0.05$). This effect is not seen with cAMP or MPA alone. (Figure 5.2)

The effects of either progestin alone or cell-permeable cAMP analog (8-bromo-cAMP) alone were minor and not significant. An exception to this was the stimulation of VEGF message by 8-Br-cAMP which was evident in the absence of progesterone. DM treatment resulted in induction of VEGF message relative to MPA alone (2.6 ± 0.8; 0.7 ± 0.1, $p< 0.01$). See Figure 5.2

Similarly PDEIV message also increased significantly with DM, but this is likely to be a cAMP effect that did not reach significance, and is comparative to MPA and not the control group (2.5 ± 0.3; 0.7 ± 0.2, $p< 0.05$) (see Figure 5.2).

Figure 5.2 *Gene expression response to decidual stimuli in pregnant CSC*



All cells were originally derived from the cervix of pregnant women (n=5). Note the increase in expression of the prostaglandin receptors EP2 (but not EP4). Also shown are changes in progesterone receptor, vascular endothelial growth factor (VEGF), phosphodiesterase type IVb, fibronectin, tissue factor and IL-8 message. Significant differences are denoted as follows: difference from control with $p < 0.01^{**}$ and $p < 0.05^{*}$, and difference from progesterone alone $p<0.01^{\Theta\Theta}$, $p<0.05^{\Theta}$.

Matrix modulators fibronectin, desmin, laminin and β-2microglobulin were not significantly up-regulated with decidualisation stimuli, as is seen in endometrial stromal decidualisation (see Figure 5.2 and Table 5.1). In pregnant CSC treated with DM, fibronectin expression was not significantly altered from control (0.66 ± 0.08 s.e.m) but was suppressed relative to progesterone (1.4±0.12 s.e.m, $p< 0.01$). Non-pregnant CSC demonstrated increased fibronectin mRNA with MPA (1.9 ± 0.28; control 1± 0.001) but a trend to relative suppression with DM (1.56 ± 0.37, ns). In summary CSC exhibit progesterone-dependent stimulation of fibronectin expression with attenuation by cAMP.

Desmin follows a similar pattern with a trend toward suppression with DM in both cell types relative to control. This only reached significance in pregnancy-derived CSC ($p< 0.05$). (table 5.1 and Figure 5.3)

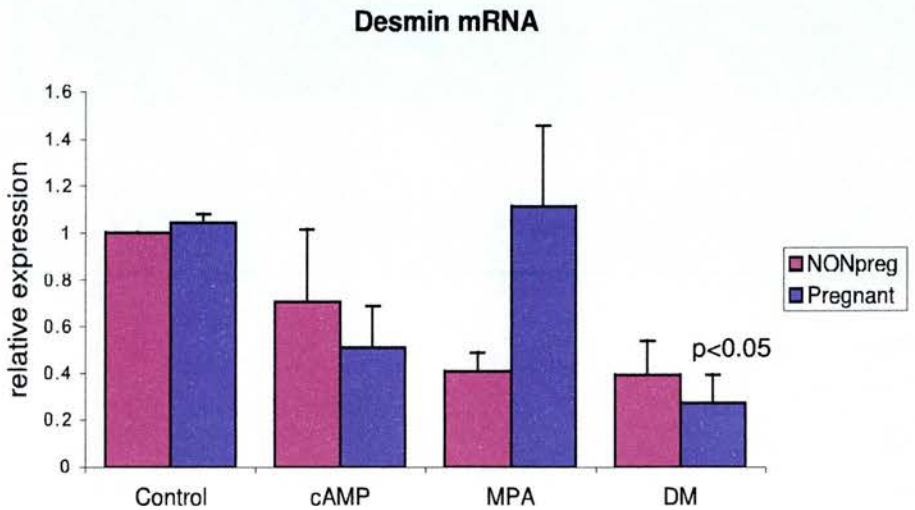
Laminin expression displays a non-significant trend to stimulation with combined DM stimuli in both pregnant and non-pregnant CSC (table 5.1).

There was no change in β-2microglobulin mRNA expression in either cell type with either cAMP, MPA or combination treatment (table 5.1).

Table 5.1 ***RNA expression response to DM (pregnant n=5, NP n=6)***

Message	Mean relative to control – sem	Significance
Cyclo-oxygenase 2	2.1 – 0.61 0.31 – 0.1 (NP)	ns ns
Cyclo-oxygenase 1	2.97 –1.14 3.26 – 0.5 (NP)	p<0.01 p<0.01
Laminin	1.4 – 0.11 1.57 – 0.24 (NP)	ns ns
Desmin	0.27 – 0.12 0.4 – 0.14 (NP)	p<0.05 ns
β2-microglobulin	0.94 – 0.19 0.81 – 0.06 (NP)	ns ns

Figure 5.3 *Gene expression of matrix modulator desmin in treated CSC*



Desmin mRNA expression after 6 day treatment period in both pregnancy and NP-derived CSC. Results expressed as mean ± s.e.m. Significance is shown in DM treated pregnancy-derived CSC relative to control, where desmin message is reduced.

5.3.2 Prostaglandin metabolism enzymes in cervical tissue and stromal cells

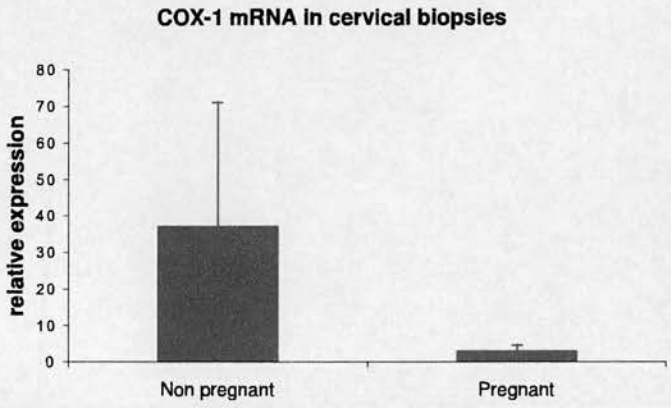
Accompanying changes in decidualisation markers seen in cervical stromal cells, were increases in agents that might maintain a high intracellular cAMP, such as an increase in COX-1. Moreover, the increase in phosphodiesterase (in this instance Type IVb) seen after stimulation with cAMP is attenuated by progesterone (Fig 5.2).

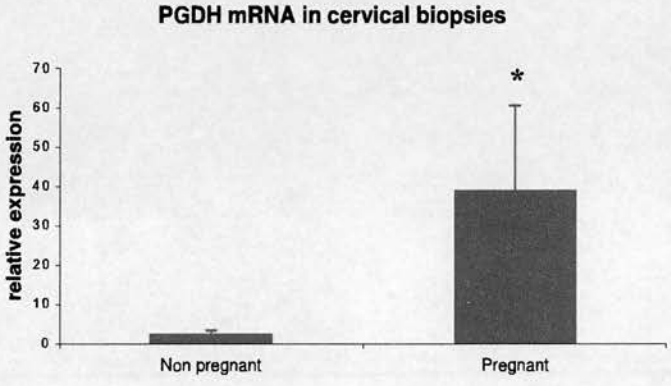
Cycloxygenase-1 is the ubiquitous PGE synthesis enzyme. COX-1 message appears to be suppressed in the cervix in pregnancy (Figure 5.4). There was a wide variation in data for all the results in the biopsy data and although the mean for the non-pregnant biopsies was 37 (s.e.m 34) compared to 2.9 (pregnant biopsies), the result was skewed by one result of 200. This again reflects the difficulties in interpreting whole tissue biopsy samples.

By comparison, in treated CSC, decidual stimuli produced a significant increase in COX-1 message in both pregnant (3 ± 1.1) and non-pregnant (3.3 ± 0.5) groups, relative to control. There were no significant changes in COX-2 (the inducible enzyme) expression in either group (table 5.1).

Prostaglandin dehydrogenase (PGDH) message was found to be increased ($p< 0.01$) in untreated pregnant cervical biopsies relative to non-pregnant cervical biopsies (mean 38.8 ± 21.7sem ; 2.3 ± 1.2sem, n=6 both groups). However during culture the PGDH message diminished and minimal message was found in treated and untreated cultured cervical stromal cells.

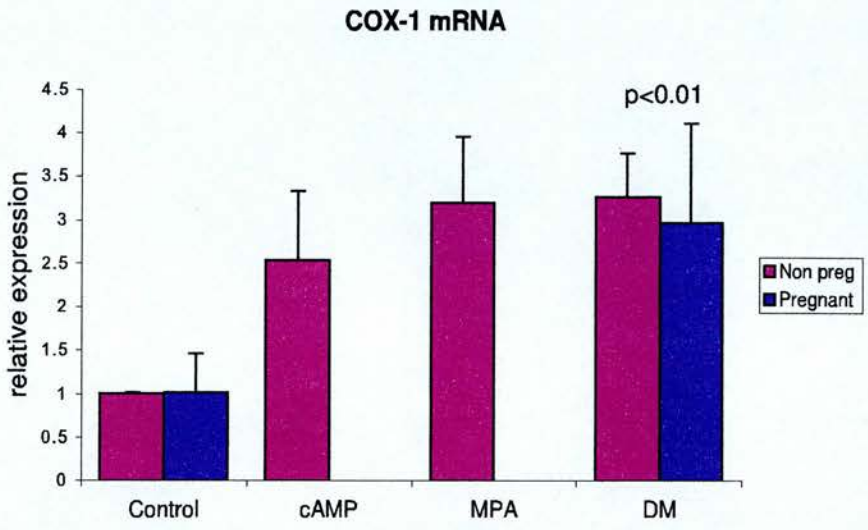
Figure 5.4 *Cervical biopsy mRNA expression in untreated samples*





mRNA expression in non-pregnant (n=6) versus pregnant (n=6) whole tissue cervical biopsies. Samples were washed and homogenised prior to RNA extraction and RT-PCR. Results are expressed as mean ± s.e.m. COX-1 and IL-8 results are non-significant due to wide variation, but PGDH* indicates $p<0.01$.

Figure 5.5 *Relative expression of COX-1 mRNA in pregnant and non-pregnant CSC*



Results expressed as relative expression to control group, mean ± s.e.m. Significance is reached for both pregnancy and NP-derived CSC after DM treatment relative to control groups in each cell type.

5.3.3 IL-8 message and protein in whole cervical tissue and stromal cells

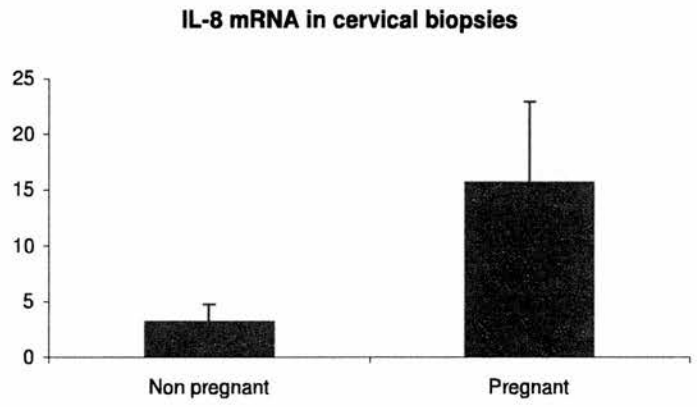
There was wide variation in IL-8 mRNA expression between individual biopsies, but there was a trend to mRNA expression up-regulation in pregnant tissue (Figure 5.6). Pooled mRNA produced a ratio of non-pregnant 1: pregnant 11, reflecting the non-significant trend seen with individual analysis (NP mean 3.2 ± 1.6 s.e.m: pregnant 15.7 ± 7.2).

This is at variance with findings from cultured fibroblasts under decidual stimuli. Combined cAMP and MPA treatment in pregnant CSC resulted in significantly decreased IL-8 message (0.39 ± 0.04, $p<0.01$), relative to control (Figure 5.2).

IL-8 protein release was also decreased by decidual stimulus of pregnancy-derived CSC; control 10.1 ± 2.8 ng/ml per 72 hours and DM 3.5 ± 1.4; $p< 0.01$ (paired data), reflecting the reduced IL8 mRNA expression in pregnant CSC. (Figure 5.7).

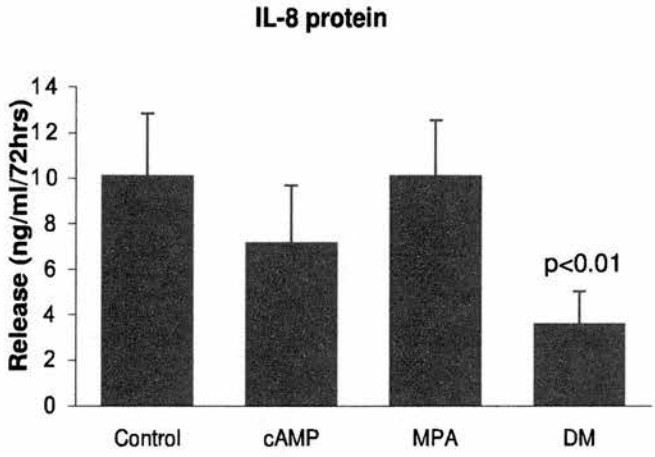
Non-pregnant CSC demonstrated no difference in IL-8 between control and all treatment arms.

Figure 5.6 *IL-8 message in untreated whole tissue cervical biopsies*



mRNA expression in non-pregnant (n=6) versus pregnant (n=6) whole tissue cervical biopsies. Samples were washed and homogenised prior to RNA extraction and RT-PCR. The y-axis depicts relative expression. Results are expressed as mean ± s.e.m and do not reach significance.

Figure 5.7 *IL-8 protein release from treated pregnant cervical stromal cells*



IL-8 protein release from pregnant CSC after a 6 day treatment period, as measured by ELISA detailed in Chapter 2. Results expressed as mean ± s.e.m. Significance is shown for DM (3.5 – 1.4 ng/ml/72hrs) relative to control (10.1 – 2.8). This mirrors the IL-8 mRNA expression in the above treatment groups, as shown in Figure 6.2.

5.3.4 Comparison of decidual stimuli

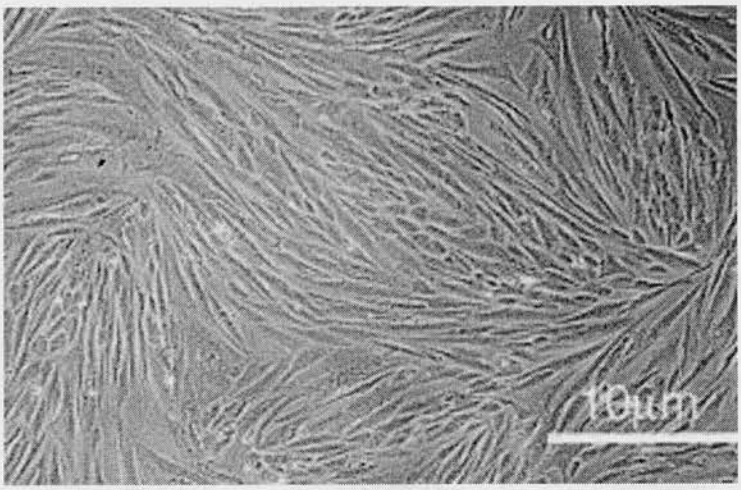
Morphology

Comparison of the PRM and DM treatment groups did reveal some differences as shown in Figures 5.8 & 5.9. Pregnant CSC treated with DM revealed a plumper morphology as shown previously, and this effect was not so marked with the non-pregnant CSC.

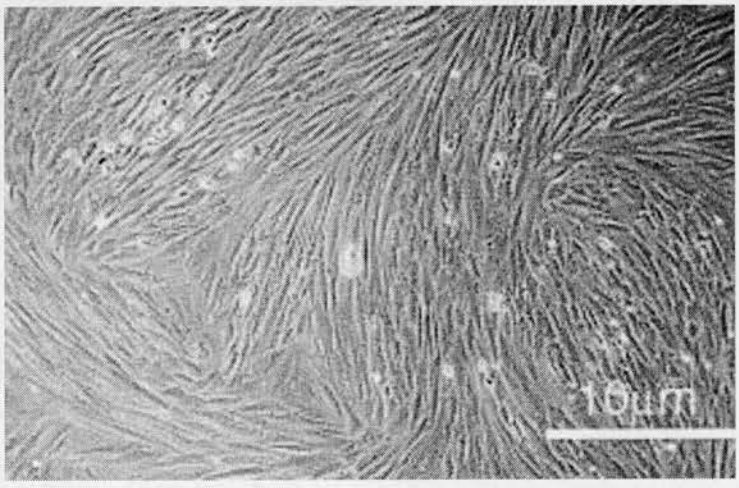
However the PRM treatment showed a more dramatic effect in the non-pregnant group, when compared with the pregnant CSC. Non-pregnant CSC exposed to PRM demonstrated an altered cell shape in a proportion of cells with visible cell projections.

Figure 5.8 *Comparison of decidualisation regimes in A) pregnant CSC*

A i)



A ii)



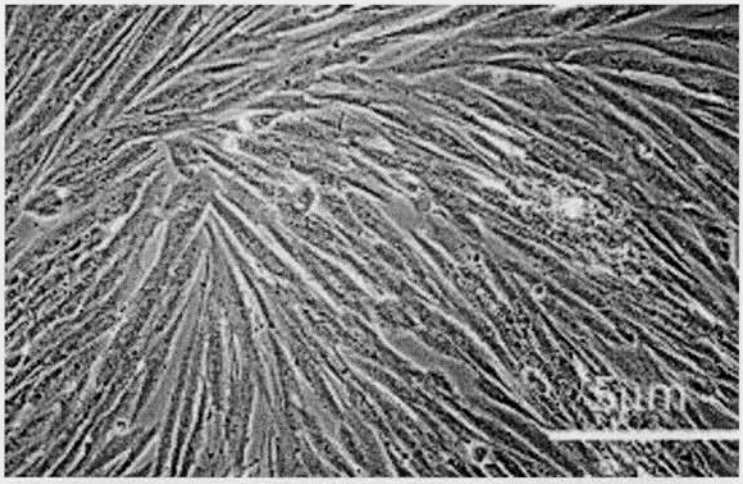
Photomicrographs (magnification x 10) of pregnancy-derived cultured CSC after 10 day treatment with either:

i) DM (cAMP, MPA and oestradiol) or
ii) PRM (PGE_2, Rolipram, MPA)

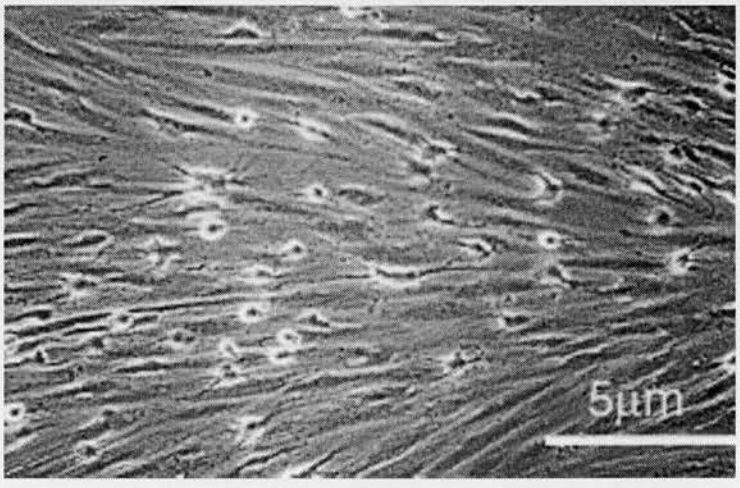
Control groups no shown here as comparison made in Chapter 5. Both DM and PRM produce similar plumper cell morphology that control groups.

Figure 5.9 *Comparison of decidualisation regimes in B) non-pregnant CSC*

B i)



B ii)



Photomicrographs (magnification x 20) of non-pregnancy-derived cultured CSC after 10 day treatment with either:

i) DM (cAMP, MPA and oestradiol) or
ii) PRM (PGE_2, Rolipram, MPA)

Control group not shown. DM produces more rounded cell morphology, but PRM stimulation produces cell projections in these non-pregnancy-derived CSC.

Gene expression

Cells derived from pregnant women differed in their decidualisation pattern from those obtained from non-pregnant women, when comparing DM with PRM (PGE + Rolipram + MPA). Both treatment regimes produced a distinct increase in IGFBP-1 mRNA expression and protein release, but to differing degrees.

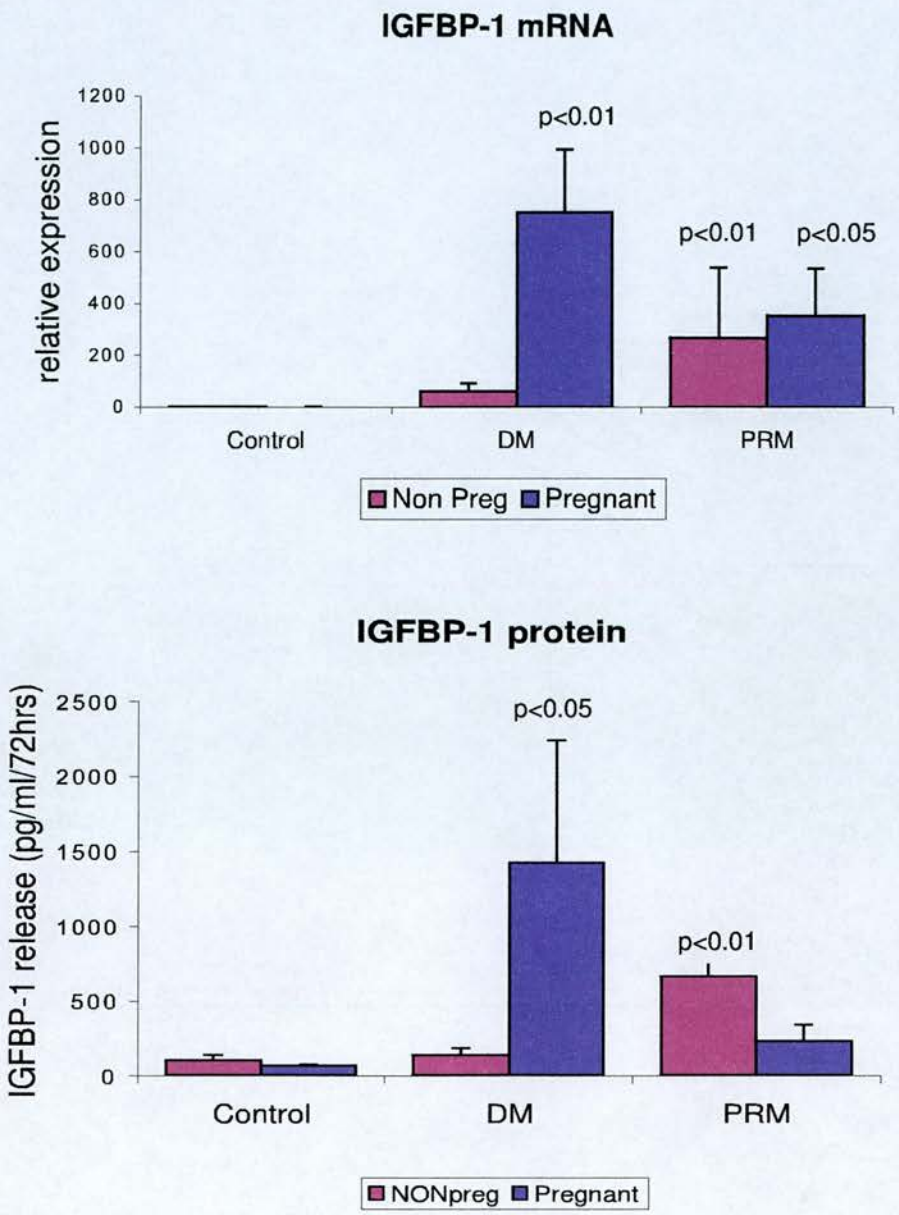
After 10 days treatment with either DM, IGFBP-1 expression was 10 times higher in the pregnancy-derived cells than in the non-pregnancy cells. With PRM treatment IGFBP-1 message increases strikingly from control, but only 1.3 times as high in pregnancy-derived cells compared to NP (Table 5.2).

Pregnant CSC demonstrated greater IGFBP-1 expression and protein release with DM, whereas the non-pregnant CSC response was more pronounced with PRM over DM. (Figure 5.12)

Table 5.2 *10 day decidualisation with different regimes, pregnant n=6, NP n=5*

Treatment	Control	DM	PGE+Rolipram+MPA
	Expression of IGFBP-1 message re lative to control (–sem)		
Pregnant	1	751±242	354±181
Non-pregnant	1	63±31	264±120
	Release of IGFBP-1 pg/ml/72 hours (–sem)		
Pregnant	66±3.5	1423±812	227±113
Non-pregnant	63	136±102	663±136

Figure 5.12 *Comparison of IGFBP-1 response to DM vs PRM treatment (Pregnant n=6, Non-pregnant n=5)*



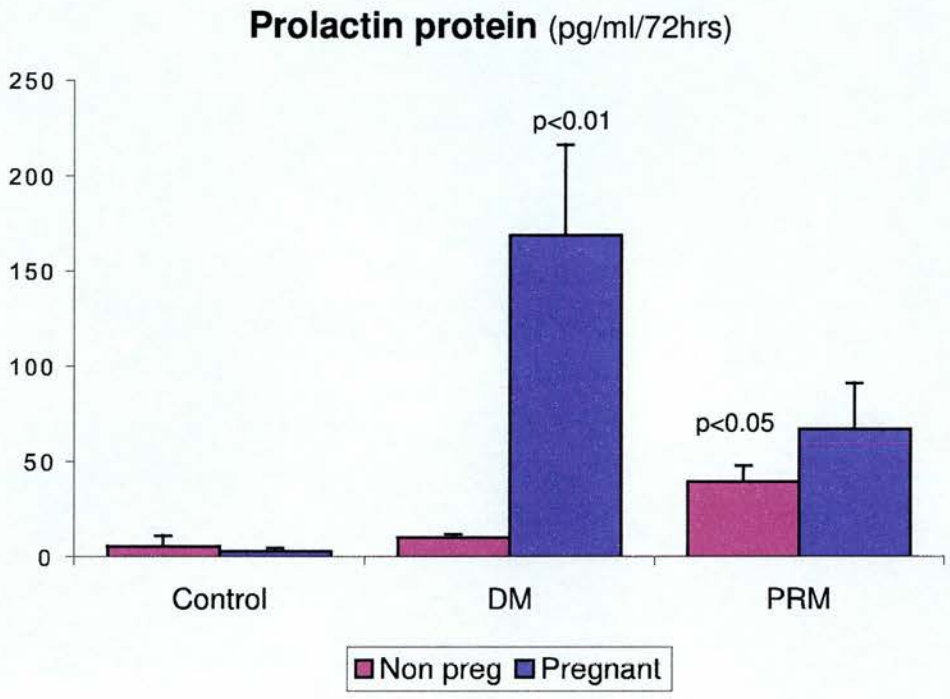
10 day treatment period. Results expressed as mean ± s.e.m. Significance expressed relative to each control for IGFBP-1 mRNA/ protein. There was no statistically significant difference in IGFBP-1 mRNA between DM and PRM groups in either pregnant/ non-pregnant CSC. IGFBP-1 mRNA is significantly increased in pregnant CSC with DM and non-pregnant CSC with PRM, relative to control (both $p<0.01$). IGFBP-1 protein release is increased relative to control ($p<0.05$) in pregnant CSC with DM, but with PRM in non-pregnant CSC ($p<0.01$). The protein release in non-pregnant CSC differed between DM and PRM ($p<0.05$).

Protein release

IGFBP-1 protein release is significantly increased with both treatment regimes.

Prolactin protein release with these two differing decidual stimuli followed a similar pattern of variation as IGFBP-1 stimulation, with pregnant CSC responding to DM but non-pregnant CSC responding to PRM. Significance was reached with DM in pregnant CSC ($p<0.01$) and with PRM in non-pregnant CSC ($p<0.05$) relative to control, but there was no statistically significant difference between the two treatment arms in either cell type, despite the visible trend. See Figure 5.13.

Figure 5.13 *Prolactin protein release from CSC in response to DM and PRM (Pregnant n=6, non-pregnant n=5)*



10 day treatment period: results expressed as mean ± s.e.m. Significance relative to individual control groups. Again a greater response is seen in pregnancy-derived CSC with DM relative to PRM, whereas non-pregnancy-derived CSC release greater quantities of decidualisation markers with PRM.

Receptor status

In non-pregnant CSC, PR mRNA expression increased significantly with PRM, but not with DM ($p<0.05$).

Pregnant CSC produce a varied response with no significant variation of PR expression between the two treatment groups (Table 5.3).

Matrix modulators

PRM stimulation down-regulates fibronectin mRNA expression ($p< 0.05$), but increases laminin ($p< 0.01$) in NP-derived CSC. IL-8 mRNA is reduced following in the non-pregnancy-derived group following PRM treatment ($p< 0.01$).

Pregnant CSC produce a varied response with no significant variation of IL-8, PR or laminin expression with either DM or PRM, but a reduction in fibronectin expression in association with DM ($p<0.05$).

Table 5.3 *mRNA expression response to DM orPRM, relative to control*

		DM *(mean ± sem)*	**PRM** (mean ± sem)	**Significance**
PR	NP	1.97 ± 0.37	**4.07 ± 1.97***	*p<0.05
	Preg	1.07 ± 0.26	6.67 ± 5.44	
IL-8	NP	0.55 ± 0.03	**0.14 ± 0.04****	**p<0.01
	Preg	1.16 ± 0.38	0.86 ± 0.27	
Fibronectin	NP	0.74 ± 0.12	**0.46 ± 0.07***	*p<0.05
	Preg	**0.52 ± 0.12***	1.31 ± 0.64	*p<0.05
Laminin	NP	3.07 ± 0.32	**3.92 ± 0.75****	**p<0.01
	Preg	1.36 ± 0.35	4.44 ± 3.19	

Overall, PRM appears to stimulate effects in non-pregnant CSC that are not seen in pregnancy-derived cell lines. The variants in this experiment are the method of cAMP elevation and origin of tissue. This confirms that there are multiple factors involved in the regulation of this decidual-like response dependent upon the means of cAMP regulation. The two methods of cAMP elevation are handled differently by pregnancy and non-pregnancy derived cells. This implies that there is some inherent difference in the response of these two cell types to specific stimuli, supporting the hypothesis that there is a phenotypic alteration of the cervical fibroblast in pregnancy.

5.4 DISCUSSION

The cervical fibroblast may change during pregnancy in response to progesterone and as the commonest cell type in the human cervix, an altered phenotype would have huge implications on cell function throughout gestation. I have shown *in vitro* evidence of decidual marker expression and protein release in cultured cervical fibroblasts exposed to decidual stimuli, with an upregulation of the requisite PR.

Further characterisation of this response supports this finding where TF and VEGF are also upregulated in CSC *in vitro*. Decidual cells under progesterone stimulation produce tissue factor (Lockwood *et al* 1993). Tissue factor has a putative role as an inhibitor of bleeding at the time of implantation and trophoblast invasion. It is known that uterine and cervical vasculature is increased in pregnancy and with proposed angiogenesis and tissue remodelling, tissue factor may be involved in regulating this. In addition to TF stimulation, VEGF message is also increased after decidual stimulus, and although this would appear to be largely a cAMP effect, significance was only reached when progestin and cAMP in combination were used. Again this may indicate some role in vascular reorganisation. In the endometrium, decidualisation is initiated perivascularly before spreading throughout the stroma, and so a similar mechanism may be suggested in the cervix although cannot be proven without immunolocalisation studies. The decidual transformation in endometrium is also associated with an increased production of the angiogenic agent VEGF and its KDR receptor (Sugino *et al* 2002) and this process may be responsible for the increased vascularisation of the cervix that is seen during pregnancy. The increase in the EP2 receptor may also facilitate VEGF release since PGE stimulates VEGF through the EP2 receptor in synovial fibroblasts (Ben-Av *et al* 1995). Although there are likely to be other cellular sources of EP receptors in the pregnant cervix, experiments in the baboon show that the EP2 type is predominant (Smith *et al* 1998) although its expression decreases during labour (Smith *et al* 2001).

Other decidualisation markers reported in endometrial stromal cells (fibronectin, desmin, laminin, β2-microglobulin) did not show significant stimulation in CSC.

This is not consistent with endometrial findings, but the very different roles of these tissues impacts on the specific functions required at various gestations and their regulation. In fact, an apparent inhibition of fibronectin was seen in CSC under decidual stimulus, and this appears to be a cAMP effect. These findings agree with those suggested by the cervical biopsy mRNA expression assessed in Chapter 4. Desmin expression was reduced in both CSC types after treatment with DM (and not cAMP or MPA alone), but only to significance in pregnancy-derived cells. Perhaps this is not surprising as the increased endometrial expression of these matrix proteins is in association with implantation, a phenomenon not normally seen in the cervix.

The assessment of PDEIV mRNA expression in pregnant CSC confirms an induction with cAMP as expected and the significant result seen with DM is likely due to variation, as no effect is seen with MPA. The effect of DM is no greater that that of cAMP, but has smaller error margins.

Prostaglandin dehydrogenase (PGDH) mRNA, the enzyme responsible for PGE_2 catabolism, is significantly increased in first trimester whole cervical tissue. Although this information must be treated with some caution in view of variation, it suggests that there may be up-regulation of the PGE catabolic pathway in the cervix in early pregnancy. Further assessment could not be obtained from this model as the PGDH message diminished in stromal cell culture and this is consistent with the paucity of data on PGDH expression in cell culture. It is possible that an alternative cell type is responsible for the PGDH expression demonstrated in the biopsy data. In whole tissue there appeared to be greater COX-1 message in non-pregnant samples but there was a wide variation and significance was not reached. The CSC model showed evidence of moderate COX-1 stimulation with combined cAMP and MPA in both cell types although, surprisingly, no changes were seen for COX-2.

Interestingly, a significant suppression in IL-8 mRNA expression and protein release is apparent after decidual stimulation in cultured cells. It could be that the phenotypic changes seen in early pregnancy differ to that which may be observed in late pregnancy, and that "decidualised CSC" have a suppressive role in terms of

immunomodulation to preserve cervical integrity. First trimester pregnancy-derived biopsy samples however revealed a trend to IL-8 up-regulation when compared to non-pregnant tissue. In particular the contradictory IL-8 result, in whole tissue compared to CSC, can be explained by the presence of other cell types capable of IL-8 expression, such as epithelial and migratory cells. This influence is then lost in isolated stromal cell culture. Progesterone suppresses IL-8 production so the culture results after prolonged progesterone exposure are to be expected (Ito *et al* 1994). IL-8 is produced by the cervix in increasing amounts during labour, is associated with chemotaxis and has been used to induce cervical ripening in animals (Barclay *et al* 1993; Chwalisz *et al* 1994; El Maradny *et al* 1994; Osmers *et al* 1995; Osman *et al* 2003; Sakamoto *et al* 2004). It is logical that IL-8 suppression throughout gestation would be an essential function of the phenotypically-altered CSC of pregnancy. Removal of this blockade in labour by the localised functional progesterone withdrawal at the receptor level in the human cervix (Stjernholm-Vladic *et al* 2004) then provides the mechanism for IL-8 production and subsequent inflammatory response of labour (Sakamoto *et al* 2004).

Moreover, when DM is compared with the prostaglandin, rolipram and progestin combination, the same changes in decidualisation markers are seen. The combination of prostaglandin and PDE inhibitor mimic cAMP administration and in concert with progesterone, provide a similar environment to that used to induce decidual changes in endometrial stromal cells. It has been shown that this combination (PRM) stimulates decidual-like changes in CSC *in vitro* studies as seen with DM. PRM produces the same induction of PRL, IGFBP-1 and PR message and protein release as shown with DM. There is also a similar pattern of fibronectin and IL-8 inhibition. Pregnancy provides high progesterone levels and localised prostaglandin changes are seen in vivo. PDEIV inhibition helps to maintain a high cAMP environment by reducing its inactivation. These influences are present in pregnancy and may provide a physiological stimulus to encourage this decidual-like transformation.

It is interesting to observe the differing response between both cell types (pregnant and non-pregnant CSC) and the two regimes used (DM and PRM). The response to DM is much more dramatic in pregnancy-derived CSC than in non-pregnancy-derived cell lines as one might expect due to duration of exposure pre-culture. However, non-pregnant cell lines appear to have an enhanced response to PRM and this suggest a differing mechanism of action or ability to respond, possibly to due to change in cellular phenotype.

Further assessment of prostaglandin metabolism, by estimation and localisation of specific enzymes and metabolites would provide essential information in evaluating the potential influence of differing cell types. There may be compartmentalised prostaglandin metabolism that would explain the differences seen in whole tissue and cell culture. Similarly, the same would apply to any factor influencing matrix modulation, inflammatory mediators or angiogenesis.

Endometrial progesterone withdrawal results in menstruation, and this raises the possibility of altered function of these cervical cells at differing stages of the menstrual cycle and pregnancy. Clearly this may involve interaction with other resident and migratory cells, but the "decidualised" fibroblast may modulate the activity of local leukocytes, facilitate communication between cells and cAMP modulators, or influence the ECM remodelling cascade or angiogenic pathway. The interaction between progesterone and prostaglandin pathways provides a physiologically plausible and self-maintaining mechanism whereby a pro-decidual influence may be present in pregnancy.

CHAPTER 6

MMP-14 IN THE HUMAN CERVIX

6.1 INTRODUCTION

Migratory inflammatory cells and matrix metalloproteinases appear to have significant roles in cervical remodelling and immune cell trafficking is evident in the cervix and myometrium throughout pregnancy.

Evidence of increased leukocyte numbers in mouse (Yellon *et al* 2003) and human uteri (Thomson *et al* 1999; Winkler *et al* 1999b) and cervices (Bokstrom *et al* 1997) have been shown in advanced pregnancy and labour (Osman *et al* 2003) as discussed in Chapter 1. In humans, the predominant migratory cells seen in the cervix are neutrophils and monocytes in association with labour, although Bokstrom identified a pre-labour leukocyte recruitment with no subsequent increase thereafter. Concurrent increases in collagenolytic and proteolytic enzymes at term have been demonstrated (Rajabi *et al* 1988; Osmers *et al* 1990) with varied identified sources of these enzymes including stromal cells, smooth muscle cells and leukocytes (Osmers *et al* 1992; Ledingham *et al* 1999a; Stygar *et al* 2002).

Some chemotactic signal is likely to trigger this neutrophil infiltration, and chemokines are produced by cervical tissue with up regulation at term and with labour (Winkler *et al* 1998; Denison *et al* 1999; Sennstrom *et al* 2000). Adhesion molecule up-regulation has also been observed (Winkler *et al* 1998; Denison *et al* 1999; Sennstrom *et al* 2000; Ledingham *et al* 2001b; Winkler *et al* 2003). The initial chemotactic signal may originate from resident cells or monocytes, which then produce cytokines and pro-MMPs thereby promoting the inflammatory cascade and contributing to extracellular matrix remodelling.

Monocyte chemotactic protein -1 (MCP-1) is a small protein beta-chemokine, chemotactic for leukocytes and secreted by differing cell types including monocytes, fibroblasts and endothelial cells. MCP-1 recruits and activates leukocytes, can influence fibroblast activity and stimulates collagen synthesis and matrix deposition during inflammatory processes and fibrosis. In human dermal fibroblasts MMP-1

and MMP-2 gene expression and protein synthesis are upregulated by MCP-1 in vitro (Yamamoto *et al* 2000). MCP-1 is produced by human cervical tissue in early pregnancy and mifepristone significantly increased its release with a concurrent increase in monocyte numbers (Denison *et al* 2000). This is a potential mechanism of action of MCP-1 in cervical remodelling as it may stimulate leukocyte migration and matrix metalloproteinase activity.

Neutrophils infiltrating cervical tissue are known to be a plentiful source of MMP-8 (Osmers *et al* 1992) and MMP-9 (Uldbjerg *et al* 1983a) whereas cervical resident cells, particularly stromal fibroblasts, have been identified as the primary sources of MMP-1 and MMP-2 in non-pregnant, early pregnant and term pregnant cervical biopsies (Ledingham *et al* 1999a; Denison *et al* 2000; Stygar *et al* 2002; Yoshida *et al* 2002). MMP-2 and -9 upregulation has been confirmed in late pregnancy (Stygar *et al* 2002) and MMP-8 with labour (Sennstrom *et al* 2003), but no conclusion can be drawn regarding cervical changes pre-labour as specific groups with ripe and unripe cervices have not been compared.

MMP cleavage is required from its pro-form for activation. MMP-14, a membrane-type MMP (MT1-MMP), mediates the activation of MMP-2 on the cell surface and is therefore essential for MMP-2 function. MMP-14 is thought to act through a prostaglandin-cAMP dependent mechanism (Shankavaram *et al* 2001). Activators of adenyl cyclase (e.g. PGE_2 via EP2 or EP4 receptors) increase ATP conversion to cAMP. PDE inhibitors such as rolipram or relaxin prevent cAMP breakdown, thereby maintaining high cAMP levels (Bartsch *et al* 2001). Phosphodiesterase enzymes promote cAMP catabolism and their inhibitors therefore maintain high cAMP levels. A combination of PGE_2 and a phosphodiesterase inhibitor (eg. rolipram; type IV PDE inhibitor) could act synergistically in the regulation of MMP-14. As both MMP-2 and MMP-8 levels are increased in pregnancy and labour then MMP-14, as a mediator of at least these two MMPs, would be expected to be present in the human cervix and up-regulated in pregnancy or labour.

Hypothesis

The hypothesis is that MMP-14 is produced by the normal non-pregnant human cervix, specifically the cervical fibroblast and that it is up-regulated by prostaglandin via a cAMP mechanism as in other cell types.

In order to assess the expression of MMP-14, and its origin within the human cervix, MCP-1 and PGE_2 treatments were compared with control in cervical explants, cultured cervical fibroblasts and cultured monocytes. Where PGE_2 was shown to have an effect, combination therapy with a phosphodiesterase inhibitor was assessed.

Aims:

1. To measure MMP-14 mRNA expression in non-pregnancy derived cervical biopsies.

2. To evaluate MMP-14 mRNA expression in cultured human cervical fibroblasts (non-pregnant and pregnant) in response to PGE_2 and MCP-1.

3. To assess the expression of MMP-14 in a monocyte cell line in response to treatment with PGE_2, MCP-1 or phosphodiesterase inhibitor type-IV, and the effect of combination therapy.

6.2 METHODS

Human cervical biopsies (n=2) were taken from non-pregnant women at the time of elective hysterectomy for benign reasons. Biopsies were divided into 2-3mm^3 explants and placed in 6 well plates, in triplicate, with 5mls of either:

1. complete medium
2. PGE_2 $1x10^{-6}$
3. MCP-1 10ng/ml.

After 24hrs the supernatant was removed, the explants were homogenised and placed in 1ml trireagent for total RNA extraction and RT-PCR.

Cultured human cervical fibroblasts (n=8, passage 2-4) from non-pregnant tissue were plated in small flasks or 6 well plates at a density of 1 x 10^5 cells /ml in complete medium and left to rest for 24 hours. The medium was then changed to:

1. control medium
2. PGE_2 $1x10^{-6}$
3. MCP-1 10ng/ml

All treatments were in duplicate for a period of 24 hours at which point the supernatant was removed and RNA extracted for MMP-14 mRNA estimation by RT-PCR.

The same was repeated with pregnancy-derived cervical fibroblasts (n=4, passage 2) with identical culture and treatment conditions.

A monocyte cell line (U937, passage 8-39) was used to study the effect of prostaglandin, MCP-1 and rolipram on MMP-14 gene expression. Cells were

cultured in complete medium and plated at a cell density of 4 x 10^5 per ml prior to treatment. These cells remain non-adherent unless differentiated to an adherent phenotype. All treatment regimes including the controls were with LPS to activate the monocytes.

Treatment regimes were as follows:

1. Control
2. PGE_2 1 x 10^{-6}
3. MCP-1 10ng/ml
4. PGE_2 + MCP-1
5. Rolipram 1μM
6. PGE2 + rolipram

The treatment period was 24 hours. U937 cells remained in suspension throughout the treatment period, were spun down to allow for supernatant collection and RNA extraction by adding 1ml tri-reagent to each pellet. RNA extraction was performed as detailed in Chapter 2. Reverse transcription was then performed prior to RT-PCR as previously described in Chapter 2.

RT-PCR was performed for MMP-14 in all the above treatment arms.

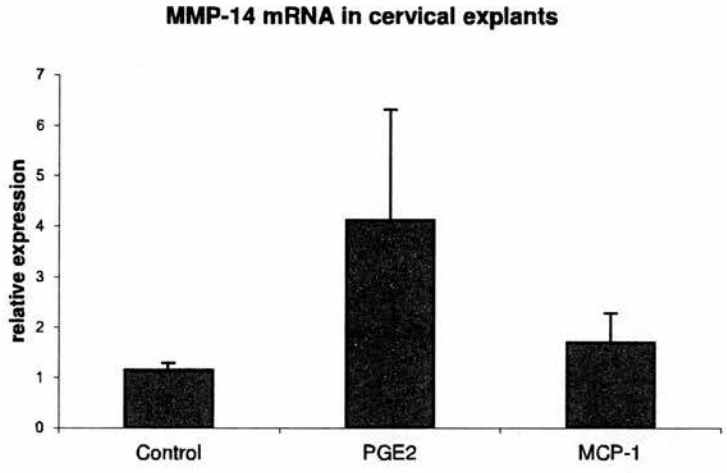
6.3 RESULTS

Human cervical explants (n=2) expressed MMP-14 mRNA after 24hr incubation with complete medium. Those treated with PGE_2 revealed a four-fold increase in MMP-14 mRNA expression (4.13 ± 2.18), but this is not significant in view of the small sample size. There was no significant change with MCP-1 treatment (1.7 ± 0.58). (Figure 6.1)

In cultured human cervical fibroblasts, PGE_2 produced no variation in MMP-14 mRNA expression relative to control, in pregnant (n=4) or non-pregnant (n=8) cell lines (0.69 ± 0.14, 0.76 ± 0.1 respectively). Incubation with MCP-1 did not alter MMP-14 expression in either cell line (1.5 ± 0.39, 0.8 ± 0.15) relative to control. (Figure 6.2)

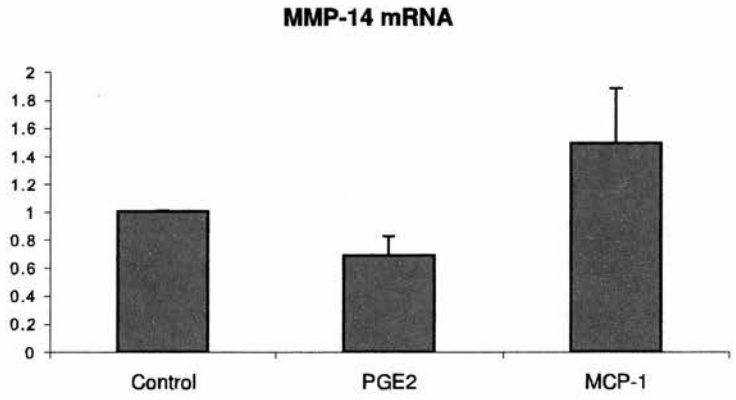
In the U937 cell model, MMP-14 mRNA expression was found to be increased above control in PGE_2 treated groups and PDEIV inhibitor groups, and an apparent although not statistically significant synergistic rise was observed in the combination group. MCP-1 treatment demonstrated no appreciable effect, and rolipram alone almost doubled MMP-14 mRNA expression (1.92 ± 0.28) but this was not significant. PGE_2 alone produced a five-fold (5.14 ± 0.55) increase in MMP-14 mRNA expression with a non-significant further increase when combined with MCP-1 (5.33 ± 0.45) or rolipram (6.44 ± 1.05). (Figure 6.3)

Figure 6.1 *MMP-14 expression in treated cervical explants (non-pregnant) n=2*



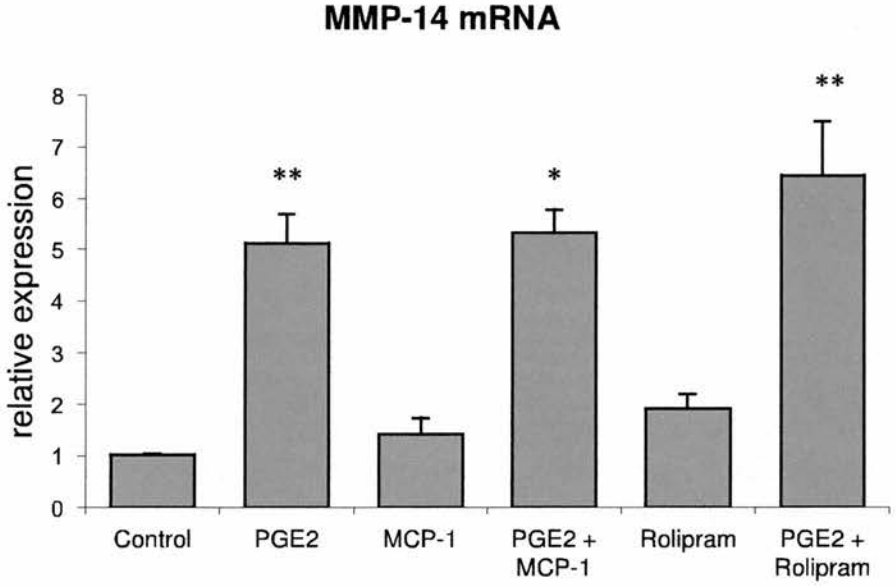
Results shown as mean ± s.e.m. Non significant increase in MMP-14 mRNA expression with PGE_2 treatment of whole tissue cervical explants.

Figure 6.2 *MMP-14 mRNA expression in cervical fibroblasts (non-pregnant, n=8)*



Results shown as mean ± s.e.m. No change in MMP-14 mRNA expression in treated cervical fibroblasts, relative to control. $p<0.05$ for the difference between PGE_2 and MCP-1 treated groups. The same was repeated for pregnancy-derived cervical fibroblasts (n=4) with no change in MMP-14 expression (PGE_2 0.77 ± 0.1, MCP-1 0.8 ± 0.15).

Figure 6.3 *MMP-14 mRNA expression in activated U937 cells*



Results shown as mean ± s.e.m. n varied for treatment groups due to availability of cultured cells: n=9 for control and PGE_2, n=7 for MCP-1± PGE_2, n=5 for rolipram, n=4 for PGE_2 + rolipram.

U937 cell MMP-14 mRNA expression was significantly increased with PGE_2 alone and any treatment group including PGE_2 relative to control, and with PGE_2 relative to MCP-1 ($p<0.05$).

** $p<0.001$ relative to control, * $p<0.05$ relative to control.

6.4 DISCUSSION

This study shows that in the context of the human cervix MMP-14 mRNA is expressed and is induced in non-pregnant cervical explants incubated with PGE_2. More specifically MMP-14 mRNA expression has been shown in cultured human cervical fibroblasts derived within and outwith pregnancy, but in this specific cell population the proposed hypothesis that PGE_2 induces fibroblast derived MMP-14 is not supported.

This implies that the PGE_2-induced MMP-14 expression must originate from migratory cells in the cervix or possibly other resident cells, such as epithelial cells. This study supports the hypothesis that the monocyte expresses MMP-14 and that PGE_2 stimulates activated monocytes to increase MMP-14 expression. These findings are in agreement with those of Shankavaram which conclude that MMP-14 expression is induced by LPS activation in monocytes, and further augmented by prostaglandin (Shankavaram *et al* 2001).

However from this data it cannot be excluded that another resident cervical cell population may be responsible for this. To do so would require further study of cervical cell populations and localisation of MMP-14 expression, ideally by quantitative immunohistochemistry. Nor can I draw any conclusions regarding gestation dependent variation in cervical MMP-14 expression as samples were only obtained in early pregnancy or outwith pregnancy. It is regretful that there was not enough pregnancy-derived tissue for the explant protocol in addition to fibroblast culture. It is perhaps of significance that there was no difference in response to PGE_2 between pregnancy and non-pregnancy derived fibroblasts. A different response might be expected if the fibroblast phenotype has changed in any way during early pregnancy, but it is also possible that unless this change is irreversible, any effect may be lost in subsequent culture.

In-vivo the PGE_2 or monocyte-associated regulation of MMP-14 is likely to be a late gestation event in the cervix and may contribute to the leukocyte infiltration and collagenolytic activity seen at term in labour. These findings emphasise the cross-talk occurring between cell types, both resident and migratory, to achieve tissue remodelling. The hypothesis is that monocyte-produced MMP-14 then activates proMMP-2 released from other resident cells to its active form MMP-2. MMP-2 is substrate specific for collagen type IV in basement membranes, where disruption will assist in further leukocyte infiltration and degranulation. With leukocyte invasion towards term, monocytes are also activated, by PGE_2, to release degradative enzymes contributing to the dissolution of ECM and collagen fibrils.

The balance between MMPs and their respective TIMP has been thought to be key in the mechanism of extracellular matrix remodelling. Since this work was done an exception to this has been identified where a surface bound form of MMP-8 on the neutrophil is not inhibited by TIMPs (Owen *et al* 2004). This then complicates the potential mechanisms at play in tissue remodelling between cell types and regulators of matrix modulation, but confirms that there are situations where the regulatory effect of TIMPs on MMP activation may be minimised.

The main source of MMP-8 is the neutrophil, but on activation by inflammatory mediators these cells release only a small proportion of their MMP-8 capacity and this is in its latent pro-form. Incubation of activated neutrophils with collagen type I (the major substrate of MMP-8) produces pericellular collagenolysis as a result of membrane-bound MMP-8, which has been shown to be resistant to TIMP inhibition. This neutrophil membrane bound MMP-8 also has enhanced stability relative to soluble MMP-8 (Owen *et al* 2004). Further exploration may demonstrate a similar mechanism with other MMPs, where soluble or membrane-bound forms of a particular MMP differ in their activity and resistance to local inhibitors. In particular, pericellular collagenase activity has been shown in the guinea-pig cervix surrounding cervical fibroblasts after treatment with antiprogestins (Hegele-Hartung *et al* 1989).

Unfortunately, it was impossible to perform other studies on pregnant cervical explants due to a limited supply of pregnancy-derived tissue, but ideally one would assess this at differing gestation and further explore sites of production with immunohistochemical studies.

MCP-1 did not appear to have a significant effect in these culture conditions or in the explant study, but none of these conditions accurately represent physiological conditions where the attraction and activation of monocytes is possible. A role for MCP-1 cannot be excluded as it is likely that the interaction between fibroblasts and monocytes is relevant. Outwith in-vivo studies, then further explant studies or co-culture with fibroblasts and monocytes would be essential to assess this aspect of cervical ripening.

PGE_2 and rolipram in combination do appear to have some synergism in monocyte MMP-14 mRNA up-regulation, but this was not statistically significant and again does not represent the physiological situation where differing cell types can be recruited and activated in unison.

It can be concluded that MMP-14 is certainly expressed in the human cervix, cervical fibroblast and monocyte. MMP-14 mRNA expression is up-regulated in monocytes by PGE_2 alone or in combination with a phosphodiesterase inhibitor. The influence of this MMP stimulation in-vivo is uncertain, but this particular MMP is known to activate MMP-2 and proMMP-8 (Holopainen *et al* 2003). These are two key enzymes released by stromal cells and neutrophils respectively, that in combination would have a significant effect on local collagenolysis. The presence of increased uterine prostaglandin and cAMP is a physiological finding toward late pregnancy and peripartum, and particularly in association with preterm labour. This proposed mechanism is pertinent in cervical remodelling as the cervical fibroblast may modulate not only collagen reorganisation but also BM degradation thereby aiding leukocyte extravasation.

Distinction of MMPs and their various forms appears to be in its infancy, as their activation mechanisms, interdependencies, substrate specificities, resistance to inhibitors and relative tissue stabilities are now seen to be extremely complex. The key role of MMPs in cervical ripening cannot be doubted, but the initiation process and relative contribution of the fibroblast and the leukocyte remains unknown. Further elucidation of the mechanisms of activation, site of action, stability and inhibition of MMPs is essential, but it must always be remembered that this is the end-stage in the cervical ripening process. Whilst for the purposes of artificial initiation of cervical ripening this may be an appropriate level to aim interventions, where prevention of abnormal preterm cervical remodelling is desirable, effective interventions may be best targeted toward prior events in the cascade of MMP stimulation.

CHAPTER 7

CONCLUSIONS

7.1 SYNOPSIS OF RESULTS AND GENERAL DISCUSSION

Within the literature review current methods of cervical ripening were reviewed and as a result of this a novel drug delivery method was assessed. Particular issues with drug administration to the cervix are the epithelial barrier, variable absorption and increased tissue density. Also the cervical site and environment preclude certain drug administration methods such as patches. With regard to cervical ripening, the proposed target is ideally the internal os (Van Meir *et al* 1997; Challis *et al* 1999) and as extracellular matrix reorganisation is a major factor (Uldbjerg *et al* 1983b; Uldbjerg *et al* 1983a), stromal tissue distribution of active drug is essential. Initial assessment of a needle-free injector device demonstrates that the device can breach the epithelium and penetrate the cervical stroma. The potential additional benefits of this are to provide a reliable dosage directly to the target area. This could reduce the administration to effect interval and the drug dosage required. It is accepted that this provides evidence by proof of principle only as this was performed on uteri removed at hysterectomy, and there remains many issues relating to clinical practicalities, clinical use, safety and patient acceptability in the context of obstetrics and gynaecology. Unfortunately due to time constraints as a direct result of delays incurred by legal issues and departmental relocation, further placebo clinical studies were postponed. Later withdrawal of the device supply for design issues made it impossible to pursue this project further and the direction of my research changed significantly at an advanced time-point in my MD program.

The focus then turned to the cervical fibroblast and it's role in pregnancy, the hypothesis being that an altered decidual-like cervical stromal cell may exist in pregnancy as is seen in the endometrium. Cervical stromal cells are exposed to the same progesterone-rich environment for a prolonged period, these cells are known to possess nuclear progesterone receptors and are responsive to progestins and antiprogestins in both animal and human models *in-vitro* and *in-vivo* (Hegele-Hartung *et al* 1989; Chwalisz *et al* 1994; Stjernholm *et al* 1999; Denison *et al* 2000; Stjernholm-Vladic *et al* 2004).

It would appear from this data that there is the potential for morphological and phenotypic alterations in the cervix and specifically the cervical stromal cell population. Whether this decidual-like transformation occurs *in vivo* is uncertain, and indeed the cervix has its own specific role in reproduction, distinct from the uterus. The propensity for CSC to differentiate in this manner may be a pre-requisite for maintenance of cervical integrity until such times as delivery is appropriate.

Pregnancy-derived cervical biopsies demonstrate an up-regulation of PRL and IGFBP-1 mRNA expression, relative to non-pregnant tissue. Evidence for in-vitro transformation to a decidual-like phenotype is shown morphologically and by induction of decidualisation markers prolactin and IGFBP-1 in cultured human CSC. This occurs in both non-pregnancy and pregnancy-derived CSC, although much more marked in the latter. These findings are seen in response to a combination of both 8-bromo-cAMP and MPA, also resulting in PR and EP2 receptor mRNA stimulation.

Tissue factor and VEGF, previously described markers of endometrial decidualisation, were also stimulated as a result of cAMP effect (Lockwood *et al* 1993; Lockwood *et al* 2002) and supports this hypothesis. Tissue factor, an inhibitor of bleeding may have a haemostatic role during proposed angiogenesis and tissue remodelling. Angiogenic VEGF message, also increased after decidual stimulus, may indicate some vascular reorganisation and indeed there is evidence that TF itself may act as an autocrine enhancer for VEGF stimulation (Lockwood *et al* 2002). This data would support a similar mechanism of perivascular initiation of decidual changes in the cervix as in the endometrium.

These effects were produced *in vitro* using a cAMP analogue that is relatively resistant to phosphodiesterase inactivation, and so to explore a possible physiological mechanism, a differing regime was assessed whereby 8-bromo-cAMP was replaced with PGE_2 and rolipram (a phosphodiesterase inhibitor). This would produce a pro-cAMP environment, which, in combination with MPA, should have the same effect. In fact, this was shown to be the case, but there were some interesting differences

observed between the pregnant and non-pregnant cell lines. Discrepancies lay in the morphological changes seen with the PRM regime and the degree of response in terms of decidualisation marker message.

Although these studies were largely in cultured cervical stromal cells where environmental influences of origin may be lost, it is possible given the evidence of stromal cell differentiation that the pregnancy-derived cells have an altered phenotype that dictates a particular behaviour distinct to that of non-pregnancy derived cells. Also, one might expect the pregnancy-derived cells to have undergone this differentiation and if it were terminal then pro-decidual culture conditions should not alter their behaviour. However, the pregnancy-derived tissue was from the first trimester and cells were cultured for 10 – 20 days without progesterone stimulus prior to the introduction of decidual stimuli. It is possible that the interruption of pro-decidual conditions for cell culture, disrupted any physiological differentiation which was later re-instigated in the decidualisation model. A similar reversion of undifferentiated phenotype on withdrawal of stimulus has been seen in endometrial stromal cells (Gellersen *et al* 2003).

The role of such a phenotypic alteration can be debated but the cervical stroma certainly undergoes tissue remodelling specific to pregnancy from an early gestation, culminating in a more dramatic stromal reorganisation around the time of delivery (Kleissl *et al* 1978; Uldbjerg *et al* 1990). The cervix must initially maintain continence of the uterus only to assist in content expulsion at the appropriate time. It is unclear what the role of a differentiated cervical fibroblast might be, the possibilities are that the major function may be to program ECM for maintenance of cervical integrity throughout gestation, or that these cells may be programmed for tissue remodelling and apoptosis toward the end of pregnancy as seen in the rat (Leppert *et al* 1994).

Montes et al have demonstrated an altered cervical myofibroblastic phenotype in pregnancy at term (Montes *et al* 2002). It is possible that these cells are representative of the decidual-like stromal cell proposed here as myofibroblastic

characteristics are shown in the endometrial DSC (Oliver *et al* 1999; Kimatrai *et al* 2003). These properties were not evaluated but include ultrastructural features of high secretory activity, and cytoskeletal markers desmin, and α-smooth muscle actin (Oliver *et al* 1999; Montes *et al* 2002; Kimatrai *et al* 2003). However, desmin expression was reduced in both CSC types after treatment with DM, which is not suggestive of this type of differentiation. Montes' findings were at term in labour so cannot be compared with the early pregnancy-derived CSC model. It is also likely that there is a phenotypic alteration throughout gestation with further differentiation in association with labour when cervical function changes radically. This has been shown in a rat model where cervical fibroblast desmin positivity is maximal at term in line with maximal plasma relaxin, both which then return to basal levels in the immediate postpartum period (Varayoud *et al* 2001).

Significant suppression of IL-8 mRNA expression and protein release is apparent after decidual stimulation in cultured cells. This differs from whole tissue where high expression of IL-8 is seen. This can be explained by the presence of other cell types, like epithelial and migratory cells, capable of IL-8 expression. However, with the focus on the fibroblast, progesterone suppression of IL-8 is well documented (Ito *et al* 1994), so it is not surprising with localised functional progesterone withdrawal at the receptor level in the human cervix (Stjernholm-Vladic *et al* 2004) to see IL-8 up-regulation in association with labour (Osman *et al* 2003; Sakamoto *et al* 2004). It is logical that IL-8 suppression should be an essential function of the phenotypically-altered CSC of pregnancy, so that removal of this blockade then provides the mechanism for IL-8 production and subsequent inflammatory response of labour.

Tornblom et al have shown a reduction in cervical stromal PGDH expression with labour and no change in COX-2 message (Tornblom *et al* 2004). This would constitute an overall increase in cervical PGE_2 peripartum that has long been suspected but has evaded confirmation. In these studies an increase in PGDH message was observed in cervical whole tissue in pregnancy although this is not comparative as these were first trimester samples it does suggest an up-regulation of PGE catabolism in the cervix in early pregnancy. Cultured CSC appeared to lose

PGDH message suggesting that the origin of this message may be from an alternative cell type. It is reasonable to expect raised PGDH in early pregnancy as a protective mechanism, with down-regulation at the time of delivery (Cheng *et al* 1993; Challis *et al* 1999; Tornblom *et al* 2004). Prostaglandin synthesis and catabolism could be further assessed by estimation and localisation studies of specific enzymes and metabolites that would provide essential information in evaluating the influence of differing cell types. It is likely that there is compartmentalisation of prostaglandin metabolism that would explain the differences seen in whole tissue and cell culture.

To speculate, the cervical fibroblast has the potential to orchestrate tissue remodelling in all stages of pregnancy, or even in menstruation but the function of the cells phenotypically altered by "decidualisation" is unclear. Gene expression and protein secretion resemble endometrial decidualisation, yet the function of this endometrial transformation is undetermined. Endometrial progesterone withdrawal results in menstruation and functional progesterone withdrawal is thought to occur pre-labour (Lockwood *et al* 1998; Allport *et al* 2001; Mesiano *et al* 2002; Stjernholm-Vladic *et al* 2004). As decidual changes are progesterone dependent, this supports the possibility of altered function of these "decidual-like" cervical cells at term.

These findings on the "decidual-like" changes in cultured cervical stromal cells open a new area for the study of the cervical changes in pregnancy. There appears to be phases of cervical alteration, as organisational changes are seen in early pregnancy as a gradual phenomenon, later replaced by the more dramatic remodelling of peri-partum cervical ripening. It has been appreciated for some time that the early stages of labour are associated with changes in the cervix, but probably the most dramatic stage of cervical remodelling occurs after delivery in restoring the cervix to its pre-pregnant state. This stage has been neglected and may provide illuminating information as the restorative processes of tissue remodelling must be highly active. The "decidualised" stromal fibroblast is a newly identified target for known initiators of ripening such as PGE_2, NO donors and in particular antiprogestins. In addition, advances in the knowledge of physiological cervical changes in pregnancy may be

exploited in attempts to prevent pathological cervical events in preterm labour. Specifically if the progesterone dependent "decidual" cervical stromal change is important for cervical integrity, then this would provide a mechanism for progesterone based PTL prevention, particularly where cervical incompetence is thought to be the primary pathology.

Finally, the role of matrix metalloproteinases in the cervix was discussed in Chapter 1 and in Chapter 6 the mRNA expression of MMP-14, a membrane-type MMP, and activator of MMP-2 and proMMP-8, was investigated with regard to cervical ripening. MMP-14 mRNA expression was confirmed by RT-PCR in human whole tissue cervical biopsies (non-pregnant), cultured cervical fibroblasts and a human monocyte cell line. Collagen degradation requires the stepwise cleavage of matrix proteins, and MMP substrates are specific. For instance, MMP-8, sourced from the neutrophil, denatures collagen type I to gelatins, a substrate for MMP-2, sourced from the cervical stromal cell. However MMP-2 is released as a latent pro-MMP and is activated by MMP-14. Cervical stromal cell MMP-2 and neutrophil MMP-9 are increased in the cervix in pregnancy at term, but do not appear to be further increased with labour (Stygar *et al* 2002). MMP-2 has substrate specificity for collagen type IV present in basement membrane. The importance of this finding is the potential role in augmenting neutrophil recruitment.

It has been shown here that MMP-14 mRNA expression appears to be up-regulated in cervical explants by PGE_2 although the sample size was small due to limited tissue supply. This up-regulation does not occur in the cervical fibroblasts, but is apparent in a monocyte cell line with significant five-fold MMP-14 message stimulation in those treated with, but not MCP-1 or rolipram (phosphodiesterase type IV inhibitor). MMP-14 production may be largely effected by leukocytes strengthening the hypothesis of proposed interaction between these and resident stromal cells to effect the ECM changes seen in cervical ripening. It is also possible that epithelial cells may be responsible for this PGE_2 dependent MMP induction. MMP-14 is membrane-bound and has wide substrate specificity for interstitial collagen types I, II and III, elastin, fibronectin, gelatin, laminin and other proteoglycans. In addition

MMP-14 activates the inactive proforms of MMP-2 proMMP-8 and MMP-13 (Holopainen *et al* 2003). The overall effect of MMP-14, and its various substrates once activated, has the potential to coordinate collagen and ECM reorganisation at several levels and this merits further assessment. MMP-14 is present in the human cervix but the cervical stromal cell is not responsible for the facilitation of leukocyte migration by basement membrane degradation as a result of PGE_2-induced MMP-14 activation of proMMP-2. There maybe other MMP interactions responsible or MMP-14 from epithelial or other migratory cells may initiate this cascade.

Valuable information could be gathered from further cervical biopsy studies throughout gestation with particular emphasis on localisation of MMP-14 by immunohistochemical means. Owen et al have demonstrated great differences in membrane-bound MMP-8 activity and inhibitor resistance compared to the soluble compound (Owen *et al* 2004) and this should also be explored for MMP-14 and others.

Any doubt over the inflammatory hypothesis of cervical changes in labour appears to be close to extinction as strong evidence gathers of the roles of cytokines, leukocyte recruitment and inflammatory mediators. Collagen re-organisation, degradation and ECM remodelling definitely occurs as a gradual process from early in pregnancy only to accelerate in the weeks, days and hours before delivery. Leukocyte ingress has been questioned but again strong evidence has now accumulated supporting this. The up-regulation of various MMPs produced by both resident cervical cells and migrated leukocytes is likely to be a response to several factors including mechanical stretch, iNOS, prostaglandin, cytokines and functional progesterone withdrawal. The only factor among these recognised to take effect in early gestation is iNOS, whereas the demonstrated effects of the others occur peri-partum. The implication here is that each regulator has its own timepoint of involvement, and if so this may help explain the varied nature of preterm labour. The gradual ECM remodelling could be augmented prematurely when this cascade is triggered.

The findings in this thesis support the hypothesis that the cervical fibroblast has a central role in cervical changes in pregnancy. I suggest that a "decidual-like" change to cervical stromal cells is present in pregnancy. The inherent properties of these cells may alter at this point and therefore affect the subsequent functions during the progressive phases of pregnancy. The role of MMPs is certain in cervical remodelling, but the timing sequence of activation is still questionable. MMP-14 in particular had not yet been identified in the pregnant cervix and I find that not only is this expressed, but is up-regulated in response to prostaglandin. However I have not been able to confirm the cellular source of increased MMP-14 production cervical tissue in these limited studies. It can only be suggested that migratory cells may have a role here.

Future studies should focus on confirmation of the existence of cervical "decidual-like" cells in-vivo. Further characterisation of this cell type will need to be repeated throughout various gestation time-points. Localisation of cell types and decidualisation markers in relation to blood vessels would be paramount in assessing this response and understanding its role. Immunolocalisation studies would best provide the required evidence. I would suggest that ultrastructural phenomena and cytoskeletal markers should provide helpful clues to localised cell function at varying stages of pregnancy and post partum. Comparison of myofibroblastic characteristics of cervical fibroblasts in response to decidual stimuli, cyclical stretch and progesterone withdrawal would indicate functional propensity in these clinical scenarios. Equally, immunolocalisation studies of cervical biopsy material would clarify cell specific MMP-14 and IL-8 production and perhaps confirm the suggestion of migratory cell activity even in early pregnancy. Clearly all this work requires cervical tissue with all the associated problems eluded to earlier. Despite this, various investigators have managed to do this and endeavours should continue. Should the phenomenon of pregnancy associated cervical fibroblast decidual-like alteration be confirmed, then the application of this cell culture model will be paramount in future studies of cervical tissue remodelling in pregnancy, labour and puerperium.

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Appendix I: Recipes for solutions

All chemicals listed were from Sigma and all dilutions were in distilled water unless otherwise stated.

1. Blocking solution

In 1 litre:

20g 2% polyvinylpyrrolidone
5g bovine serum albumin
1ml presevatives — Boehringer Mannheim
1.9g EDTA (5mmol/l)
6.1g Tris (50mmol/l)

2. Complete medium (cRPMI)

500ml RPMI 1640 supplemented with:

10% FCS
20µg/ml gentamicin
100 IU/ml penicillin
100µg/ml streptomycin
2mM L-glutamine

3. 2% FCS medium

500ml RPMI 1640 supplemented with:

2% FCS
20µg/ml gentamicin
100 IU/ml penicillin
100ug/ml streptomycin
2mM L-glutamine

4. ELISA assay buffer

For 1 litre: 9g NaCl (150mmol/l)

12.1g Tris (100mmol/l)

300ul phenol red solution (50mmol/l)Flow Laboratories

0.7g EDTA (2mmol/l)

1ml preservatives

2g BSA (2mg/ml)

pH 7.2

5. ELISA buffer and tween

As ELISA buffer above with:

0.05% Tween-20

6. ELISA Coating solution

400mM $NaHCO_3$

40mM Na_2CO_3

pH 9.6

7. ELISA Substrate

10mls 100mM sodium acetate

1ml 2g/l tetramethyl benzidine in DMF

1ml 6g/l urea-hydrogen peroxidase (in 50mM Na acetate)

pH 6.0

(100mM Na acetate: in 1litre - 13.6g sodium trihydrate, 1ml preservatives)

8. ELISA wash buffer (x 20 conc)

In 2 litre: 20mls Tween-20

360g NaCl

48.4g Tris

pH 7-7.5

At x 1conc: 9g/L NaCl (150mmol/l)

1.21g/L Tris (10mmol/l)

0.5mls Tween-20 (0.05%)

pH 7-7.5

9. Freeze mix

In 50mls FCS: 5mls (10%) DMSO

10. Methyloximating solution

10mg/ml methyoxyamine hydrochloride

10% ethanol

1M sodium acetate

pH 5.6

11. Neutral buffered formalin

In 1 litre: 6.5g Na_2HPO_4 (50mM)

4.5g $NaH_2PO_4.2H_20$ (25mM)

100ml 40% formaldehyde

900mls distilled water

12. PBS & Tween

In 1 litre PBS: 8g NaCl

100µl Tween-20

pH 7.4-7.6

13. Preservatives

200mg/ml 2-methylisothiazolone

200mg/ml bromonitrodioxane

In DMF/ DMSO 1:1

Dilute 1:1000 to use

14. 0.1M Sodium citrate buffer

In 1 litre: 29.4g Tri-sodium citrate

0.1g sodium azide

pH 6.0

Dilute 1:10 to use

15. TE buffer

10mmol/l Tris pH 8.0

1mmol/l EDTA in DEPC water

16. Tris buffer

In 1 litre: 121.1g Trizma base

pH 7.2

Appendix II: Conference Proceedings

Poster presentations:

Cowan S, Calder A.A. and Kelly R.W. Decidualisation of cervical stromal cells. Combined Blair Bell and Munro Kerr Society Meeting (June 2003), Glasgow, UK.

Appendix III: Publications

Cowan S, Calder A.A, Kelly R.W. (2004) "Decidualisation of cervical stromal cells" European Journal of Obstetrics and Gynaecology and Reproductive Biology **114**(2): 189-196

Cowan S, Calder A.A, Kelly R.W. (2003) "Decidualistion of cervical fibroblasts", in Abstracts presented at a joint meeting of the Blair Bell and Munro-Kerr Research Societies 4-5th June 2003, at the Glasgow Royal Infirmary, BJOG 110(10): 958-963.

Cowan S, Calder A.A. (2004) Parturition and the clinical interruption of pregnancy, in Curtis-Prior P.B

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Decidualisation of cervical stromal cells

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Abstract

Objective: Control of cervical function is poorly understood. The major structural component of the cervix is collagen and peri-partum cervical changes are largely due to the action of collagenase, either released by resident cells or derived from an influx of neutrophils. More importantly, the cell type that initiates the changes in the cervix is unknown although the resident fibroblast is a possible contender. Little is known about the state of the cervical fibroblast during pregnancy. Decidualisation of the endometrium is essential for implantation and pregnancy. In man, pre-decidual and decidual transformation of endometrial stroma occurs under the influence of progesterone. Decidualisation can also be induced in vitro in endometrial fibroblast-like stromal cells where the process is also dependent on elevated intracellular cAMP levels. *Study design:* Cultured human cervical fibroblasts were treated with progestin (medroxyprogesterone acetate) and cAMP elevating agents for 6 and 10 days. *Results:* After 6 days they expressed and released IGFBP-1 and prolactin (PRL) and underwent morphological changes by 10 days. In addition, there was an increase in progesterone receptor and prostaglandin E type 2 receptor mRNA (but not type 4). *Conclusion:* The propensity of cervical stromal cells to decidualise suggests that these differentiated cells may be a better model with which to study the initiation of labour.

Keywords: Decidualisation; cAMP; Cervix; IGFBP-1; Progesterone

1. Introduction

Decidualisation of the superficial endometrium is an essential process in preparation for implantation and pregnancy. This occurs in the stromal compartment during the late luteal phase and is coordinated by progesterone-dependent genes. This process must prepare either for trophoblast invasion or menstruation and therefore disturbance of this mechanism may result in menstrual disorders or subfertility. Decidual cells are terminally differentiated endometrial cells and form the main elements of decidual stroma together with bone-marrow-derived uterine NK cells. The decidual reaction, which occurs in vivo under the influence of progesterone, occurs more readily in vitro with endometrial stromal cells sensitised to progesterone by elevated cAMP levels [1,2]. The decidual phenotype is recognised by a rounded morphology and by characteristic gene expression and protein production [3,4]. Prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) are both expressed and secreted by decidual cells and so have been used as markers of decidualisation.

One of the unresolved mysteries of labour is that of cervical ripening, the process where the fibrous cervix dramatically softens and loses rigidity prior to childbirth. The nature of cervical tissue was well described by Danforth in 1947 [5]. Distinct from the muscular uterine corpus, the cervix is a fibrous organ with less than 15% of its content contributed by smooth muscle, mostly found peripherally and increasingly abundant nearer to the corpus. The remaining extracellular matrix consists of dense collagen (66% type I, 33% type III) fibril bundles and elastic fibres with intervening ground substance. Ground substance is composed of proteoglycan complexes, consisting of various glycosaminoglycan (GAG) side chains on core proteins linked to a hyaluronic acid chain. These complexes bind tightly, investing the collagen fibrils and providing rigidity. Cervical ripening has often been compared to an inflammatory reaction with increased vascularity and enzymatic

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collagen degradation [6,7] effected through matrix metalloproteinases [8,9]. Increasingly, collagen breakdown in the cervix at term is associated with an ingress of leucocytes [10–12]. The stromal fibroblast is the most likely source of collagen production in the cervix, and in addition these cells may have a key role in cervical remodelling [13] as a source of chemokines, prostaglandins, and other inflammatory mediators known to contribute to cervical ripening. If these stromal cells differentiate to a decidual phenotype, a new appraisal of their role in pregnancy and in ripening of the cervix is appropriate.

Human cervical stromal cells (CSC) also express progesterone receptor (PR) as shown in lower uterine segment stromal cells [14] and as such may play a role in changes in the cervix seen during pregnancy, such as the increased vascularisation. Although the human cervix softens at term, when circulating progesterone levels remain high, progesterone receptor antagonists do initiate changes [12] and "progesterone withdrawal" may be an effect of alteration in PR status or function. We show here that both pregnant and non-pregnant cervical fibroblast-like stromal cells respond to progestins in the presence of cAMP by undergoing "decidual" changes as seen in endometrial stromal cells.

2. Materials and methods

2.1. Tissue collection

Cervical stromal cells were isolated from both non-pregnant ($n = 6$) and pregnant ($n = 6$) cervices. Non-pregnant samples were obtained immediately post-operatively from pre-menopausal women undergoing total abdominal hysterectomy for benign pathology in the Department of Obstetrics and Gynaecology, Royal Infirmary of Edinburgh. Written informed consent was obtained, as was approval by the Lothian Research Ethics Committee and the Lothian University Hospitals NHS Trust. A thin (~2 mm) section was cut longitudinally through the anterior lip of the uterine cervix and placed in RPMI 1640 medium (Sigma, Poole, Dorset, UK) on ice for transport.

Pregnant women (gestation 7–11 weeks) attending for first trimester surgical termination of pregnancy were recruited from the out-patient clinic and written informed consent obtained, with Ethics Committee and Trust approval as above. All required a negative smear history and were chlamydia negative. A cervical biopsy was taken from the anterior lip of the cervix immediately prior to termination, using Shumaker biopsy forceps and placed in RPMI 1640 on ice for transport.

A foreskin fibroblast cell line (HS27) (European Collection of cell Culture; http://www.ecacc.org.uk) was used to compare the decidualisation response with non-reproductive stromal cells. These cells were cultured and treated in and identical manner to the cervical stromal cells.

2.2. Tissue culture

The samples were washed twice with phosphate buffered saline (PBS, Sigma, Poole, UK) and dissected into small fragments with scalpel blades. Tissue was then placed in 25 cm^2 tissue culture flasks in culture medium {RPMI 1640 supplemented with 10% FCS (Mycoplex, PAA Laboratories, Teddington, UK), 20 μg/ml gentamicin (Sigma)}, 100 IU/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 2 mM L-glutamine, at 37 °C 95% air: 5% CO_2 incubation. This resulted in outgrowth of cervical stromal cells and with medium exchange every 3–4 days the cervical stromal cells grew to confluence and were then transferred into 75 then 162 cm^2 flasks and grown to confluence within 21 days.

Where appropriate cells were frozen in liquid nitrogen using a cryopreservative medium (FCS with 10% dimethylsulfoxide (DMSO). On retrieval, cells were recovered in complete medium and grown to confluence within 7–11 days. All cells used were from passage 2–7.

Both pregnant and non-pregnant samples were also homogenised in Tri reagent (Sigma, Poole, Dorset) for RNA extraction from untreated cervical biopsy tissue.

2.3. Treatment regimes

Cells were plated at 5×10^4/ml complete medium in 6 well plates and grown to confluence over 72 h before treatment commenced. After a PBS wash, treatments consisted of: (1) 2% FCS supplemented RPMI as control (with 20 μg/ml gentamicin, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) or 2% FCS containing (2) 8-bromo-cAMP (BrcAMP) 0.1 mg/ml, (3) medroxyprogesterone acetate (MPA, 6α-methyl-17α-hydroxyprogesterone acetate) 1 μM, or (4) Decidualising Mix (DM: BrcAMP, MPA and oestradiol 1×10^{-7} M). Treatments lasted for 6 or 10 days with medium changes every 3 or 4 days. Medium was collected in duplicate at the end of each treatment period and stored at −20 °C.

A control experiment to assess oestradiol (E) effect was performed comparing control, DM and BrcAMP plus MPA over a 6 day treatment period in pregnant CSC ($n = 4$).

HS27 cells (foreskin fibroblasts derived) were cultured and treated as above (at passage 27) and control, BrcAMP and decidualising treatment were compared.

To assess the decidualisation effect of differing regimes a comparison experiment, with DM versus PRM (prostaglandin 1 μM + rolipram 1 μM + MPA 1 μM), was performed in both pregnancy and non-pregnancy derived cells. Rolipram is a type IV phosphodiesterase inhibitor and acts synergistically with prostaglandin to increase cAMP levels. These treatments were for 10 days and assessed IGFBP-1 mRNA expression and protein release.

2.4. Real-time RT-PCR

Total RNA was extracted using Tri Reagent and Phase-lock tubes (Eppendorf, Merck) according to manufacturers'

Table 1
Primer/probe sequences for real-time PCR measurements

	Forward primer	Reverse primer	Probe
EP2	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA	CCACCCTGCTGCTGCTTCTCATTGTCT
EP4	ACG CCG CCT ACT CCT ACA TG	AGA GGA CGG TGG CGA GAA T	ACG CGG GCT TCA GCT CCT TCC T
Progesterone-receptor	CAGTGGGCGTTCCAAATGA	TGGTGGAATCAACTGTATGTCTTGA	AGCCAAGCCCTAAGCCAGAGATT-CACTTT
Prolactin	GCC CCG GAG GCT ATC CTA	TCA GCT CCA TGC CCT CTA GAA	CCA AAG CTG TAG AGA TTC AGG AGC AAA CCA
IGFBP-1	CACAGGAGACATCAGGAGAAGAAA	ACACTGTCTGCTGTGATAAAATCCAT	TTCCAAATTTTACCTGCCAAACT-GCAACAA
vEGF	TACC TCCACCATGCCAAGTG	TAGCTGCGCT GATAGACATC CA	ACTTCGTGAT GATTCTGCCC TCCTCCTT
Desmin	GGAGAGGAGAGCCGGATCA	GGGCTGGTTTCTCGGAAGTT	TCTCCCCATCCAGACCTACTCTGCCC
Fibronectin	TGTTCGTGCAGCTGTTTACCA	TCTGTGACACAGTGGCCATAGG	CGCAGCCTCACCCCCAGCC
β2-Microglobulin	GGCACGAGCCGAGATGTC	CCTCCAGGCCAGAAAGAGAGA	CGCTCCGTGGCCTTAGCTGTGC
Tfactor	CAC CGA CGA GAT TGT GAA GGA	CCC TGC CGG GTA GGA GAA	TGA AGC AGA CGT ACT TGG CAC GGG T
Laminin	CCAGCCTTTCCTACGCCC	AAAGAGCTGGTTCTGATTCTGCA	CCCCAGTTCTGACACATGGTCCTTGTG
IL-8	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG	CCTTCCTGATTTCTGCAGCTCTGT-GTGAA

instructions followed by precipitation with isopropanol. All RNA samples were quantitated by UV spectrophotometry and diluted to 100 ng/μl. Four microgram of total RNA was reverse transcribed (AB Applied Biosystems Taqman Reverse Transcription Reagents, Warrington, UK according to manufacturer instructions), the resulting single-stranded cDNA was then analysed by quantitative real time Taqman PCR using a Model 7700 Sequence Detector (Perkin Elmer Applied Biosystems).

Primers and probes were designed using the Primer Express© program (AB Applied Biosystems). Sequences used are shown in Table 1. BLAST nucleotide database searches were performed for all primer/probe sets confirming specificity. All primers and probes were validated for linearity of response (ABI Prism 7700 sequence detection system manual). The slope of dilution curves was <0.1 in all but tissue factor, which was −0.18. The PCR reactions were run on a Model 7700 Sequence Detector (Perkin Elmer Applied Biosystems) in duplicate, and 18-S was used as an internal endogenous control to normalise variations in cDNA content between samples. To determine genomic DNA contamination the β-actin signal was measured without reverse transcription in all samples. The criterion for exclusion was a measurement greater than 3 standard deviations below the mean, from population of 66 samples; this translates to a β-actin FAM signal with a Ct below 27 cycles. Samples used had a mean β-actin FAM Ct of 35.9 ± 2.99 (S.D.) without reverse transcription, and the minimum in the sample set used was 29.5.

To examine the gene expression of known decidualisation markers, quantitative RT-PCR was carried out for Prolactin (PRL), IGFBP-1 (insulin-like growth factor-binding protein-1), desmin, laminin, fibronectin and β_2-microglobulin, tissue factor (TF), Interleukin-8 (IL-8) and vascular endothelial growth factor (vEGF). Changes in receptor expression were evaluated for progesterone receptor (PR) and prostaglandin E receptor (EP) types EP2 and EP4.

2.5. Enzyme linked immunosorbent assays

Release of PRL and IGFBP-1 protein from cultured cells was evaluated in medium collected at the end of any treatment period. All samples were assayed in duplicate.

PRL estimation was performed using a time-delayed fluorescence PRL kit (Perkin Elmer Applied Biosystems) according to manufacturer instructions but, in order to attain sufficient sensitivity, a 10-fold increase in sample volume relative to standard volume was used. The analytical sensitivity is 40 pg/ml and the within assay variation was 7.5% (relative standard deviation) evaluated on pooled stromal cell samples. All samples were analysed in the same assay.

The IGFBP-1 assay was performed with matched pairs of capture and biotinylated labelled detection antibodies (R&D Systems, UK) according to manufacturer instructions with a lower limit of sensitivity of 60 pg/ml and a within assay variation of 1.7% (relative standard deviation). All samples were measured in the same assay.

IL8 production was assayed by ELISA with matched pairs of capture and biotinylated labelled detection antibodies for IL-8 (R&D Systems, UK) as previously reported [15].

2.6. Fluorescence activated cell analysis

Cervical stromal cells were grown to confluence and treated with trypsin EDTA, washed and resuspended in PBS/BSA medium. The cell suspension was treated with monoclonal anti-fibroblast antibody (Oncogene Research products, Boston, MA) which recognises a fibroblast specific surface antigen. Cells were then washed and treated with fluorescein labelled sheep-anti-mouse serum. Cells

were analysed in a Beckman Coulter instrument and fluorescence intensity was plotted against number of events.

2.7. Statistics

Data normalised to control was analysed by non-parametric methods. The Kruskal–Wallis analysis of variance was used and Dunn's multiple comparisons test was used to assign significance to treatment. For comparisons of mRNA in pregnant or non-pregnant cervical biopsies, the Mann–Whitney *U*-test was used.

3. Results

Spindle-like fibroblast cells grew out of cervical tissue from both pregnant and non-pregnant women. The fibroblast identity of these cells was confirmed by FACS analysis of fibroblast surface antigen. The percentage of cells that possessed this marker was between 95 and 98% of the total (Fig. 1).

Cells treated with DM for ten days had a more rounded morphology than untreated cells cultured in the same way (Fig. 2), a change characteristic of "decidual" transition. After 6 days, cells responded to stimulation with DM by both expressing prolactin and IGFBP-1 mRNA and releasing these proteins (Fig. 3). All cervical stromal cell results discussed are from pregnant women unless otherwise stated. A similar pattern of decidualisation was seen in cells derived from non-pregnant cervices (Fig. 3).

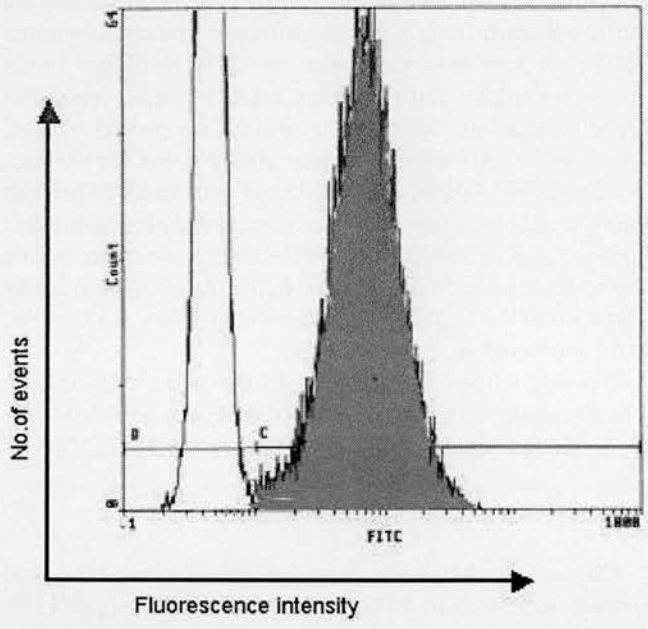

Fig. 1. Fluorescence activated cell analysis of un-differentiated cells to confirm their fibroblast nature. *Y*-axis is number of events and *X*-axis shows fluorescence intensity denoting specific fibroblast marker. Positive cells are shown in grey. Cells with the fibroblast marker constituted 98% of the cells.

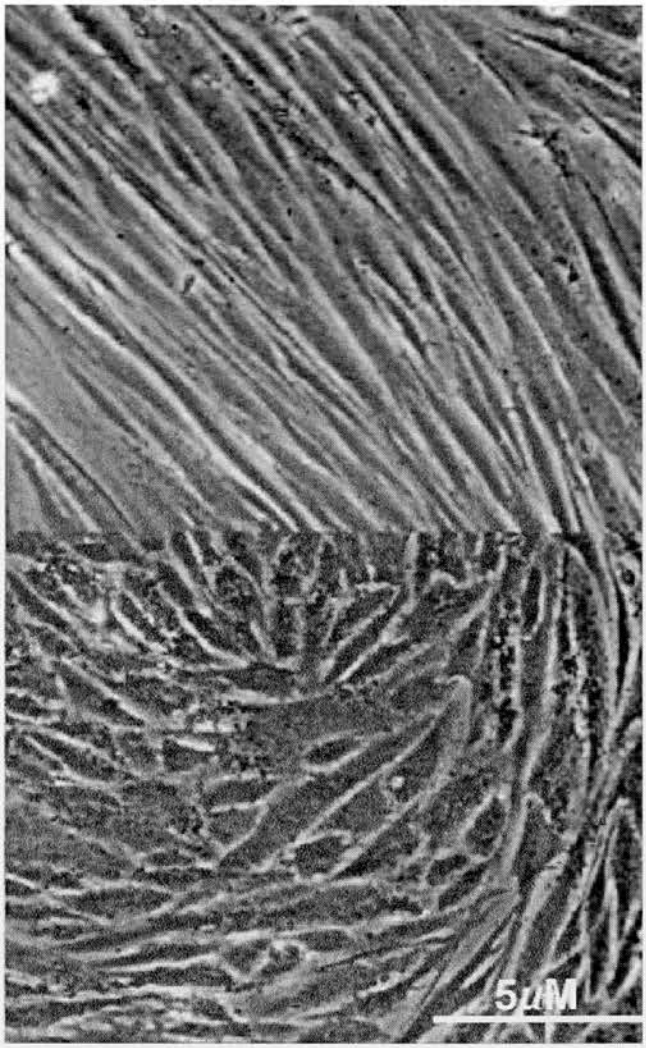

Fig. 2. Cervical fibroblasts grown in (A) control culture and (B) the same cells grown for 10 days in the presence of BrcAMP and progestin (MPA). Scale bar is 5 μM.

DM treatment also resulted in significantly increased TF mRNA expression ($P < 0.05$) and decreased IL-8 mRNA ($P < 0.01$), relative to control (Fig. 4). IL-8 protein release was also decreased by decidual stimulus (mean ± S.E.M); control 10.1 ± 2.8 ng/ml/72 h and DM 3.5 ± 1.4 ($P < 0.01$, paired data).

The effects of either progestin alone or cAMP analog (BrcAMP) alone were minor and not significant. An exception to this was the stimulation of vEGF mRNA by BrcAMP which was evident in the absence of progestin (Fig. 4).

The comparison of DM (BrcAMP, MPA, E) with BrcAMP + MPA in pregnant cervical stromal cells confirmed a significant increase in IGFBP-1 mRNA (mean fold increase in expression 253 ± 85 S.E.M.) and PRL mRNA (16 ± 7.8) with DM relative to control (both $P < 0.05$). However there was no significant difference between DM and BrcAMP + MPA treatment groups, excluding oestradiol as the significant stimulus.

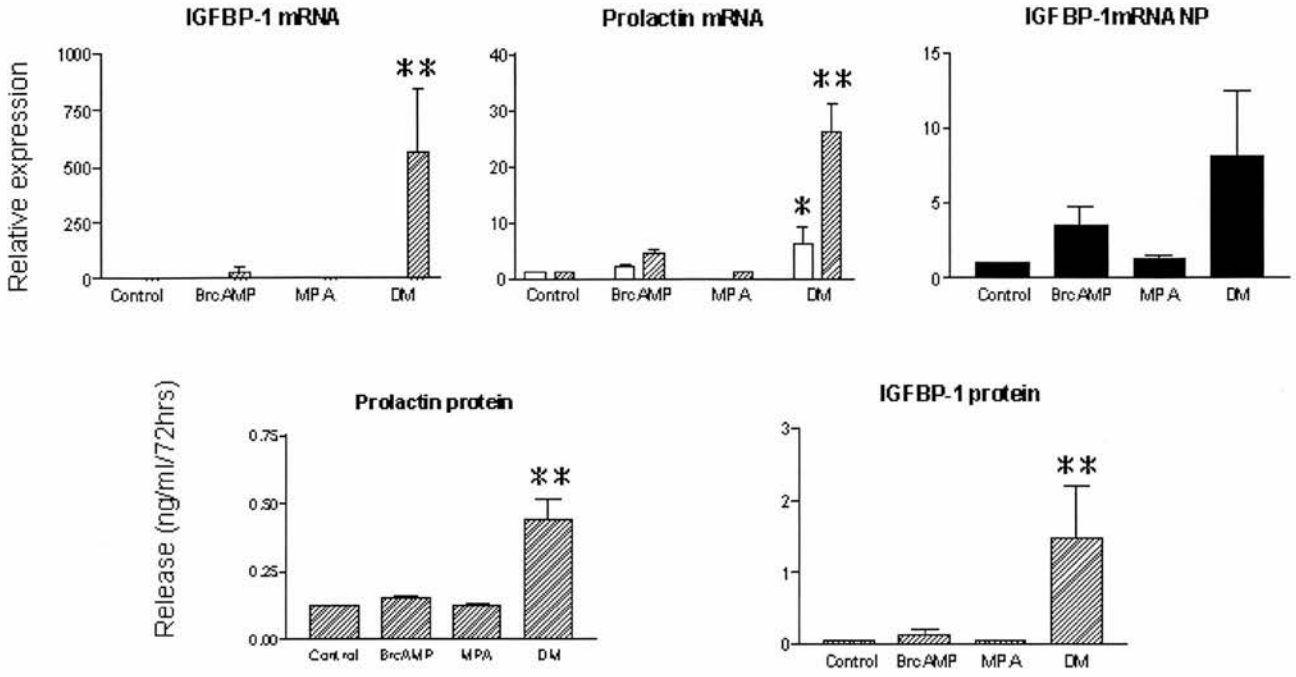

Fig. 3. The increase in expression of IGFBP-1 with combined progestin and cAMP analog. Changes in mRNA expression/protein release after 6 days differentiation with BrcAMP and a progestin, medroxyprogesterone acetate (MPA) or a combination of the two with oestradiol, decidualising mix (DM). Pregnant CSC are shown by hatched bars, NP CSC by solid bars and FF by open bars. Bars represent the mean of each treatment group ± S.E.M. Top three panels: IGFBP-1 mRNA and PRL mRNA in cells derived from the pregnant cervix ($n = 5$) and foreskin fibroblast cell line ($n = 4$). IGFBP-1 mRNA in cells from non-pregnant cervix ($n = 6$). In the lower two panels: released IGFBP-1 protein and prolactin are shown as measured by ELISA in culture medium (both from pregnancy derived samples, $n = 5$). Although progestin is without effect alone, it is essential for the expression of decidualisation markers. Significant differences are denoted as follows: difference from control with $^{**}P < 0.01$ and $^{*}P < 0.05$.

Accompanying changes in decidualisation, were increases in agents that might maintain high intracellular cAMP, such as the EP2 mRNA (Fig. 4) and a significant increase in COX-1mRNA (Table 2).

The decidualised cells showed an increase in progesterone receptor mRNA (Fig. 4). Moreover, the increase in phosphodiesterase mRNA (in this instance Type IVb) seen after stimulation with BrcAMP is attenuated by progesterone (Fig. 4).

HS27 fibroblasts did not produce the same response to decidual stimulus in identical culture and treatment conditions with no significant change in IGFBP-1 mRNA and a significant, though much less marked, increase in PRL mRNA (Fig. 3). Nor did DM produce a significant increase in progesterone receptor mRNA expression in this foreskin fibroblast cell line.

Cells derived from pregnant women differed in their decidualisation pattern from those obtained from non-pregnant women. After 10 days treatment with DM, IGFBP-1 mRNA expression was 10 times higher in the pregnancy-derived cells than in the non-pregnancy cells, but only 1.3 times as high when decidualisation was induced by PGE + Rolipram + MPA (Table 3). This confirms that PGE_2 and rolipram can mimic the effects of cAMP, in combination with MPA, but there was a difference in magnitude of effect with a greater response to DM in pregnant CSC, but a greater effect in non-pregnant CSC with PRM. This was shown for both IGFBP-1 mRNA and protein release.

Table 2
RNA expression in response to decidual stimulus (pregnant, $n = 5$)

Message	Mean relative to control ± S.E.M.	Significance
Cyclo-oxygenase 2	2.1 ± 0.61	NS
Cyclo-oxygenase 1	2.97 + 1.14	$P < 0.01$
Laminin	1.4 ± 0.11	NS
Desmin	0.27 ± 0.12	$P < 0.05$
β2-Microglobulin	0.94 ± 0.19	NS

Table 3
10 day decidualisation with different regimes, pregnant $n = 6$, NP $n = 5$

Treatment	Control	BrcAMP + MPA	PGE + Rolipram + MPA
	Expression of IGFBP-1 message relative to control (±S.E.M.)		
Pregnant	1	751 ± 242	354 ± 181
Non-pregnant	1	63 ± 31	264 ± 120
	Release of IGFBP-1 pg/ml/72 h (±S.E.M.)		
Pregnant	66 ± 3.5	1423 ± 812	227 ± 113
Non-pregnant	63	136 ± 102	663 ± 136

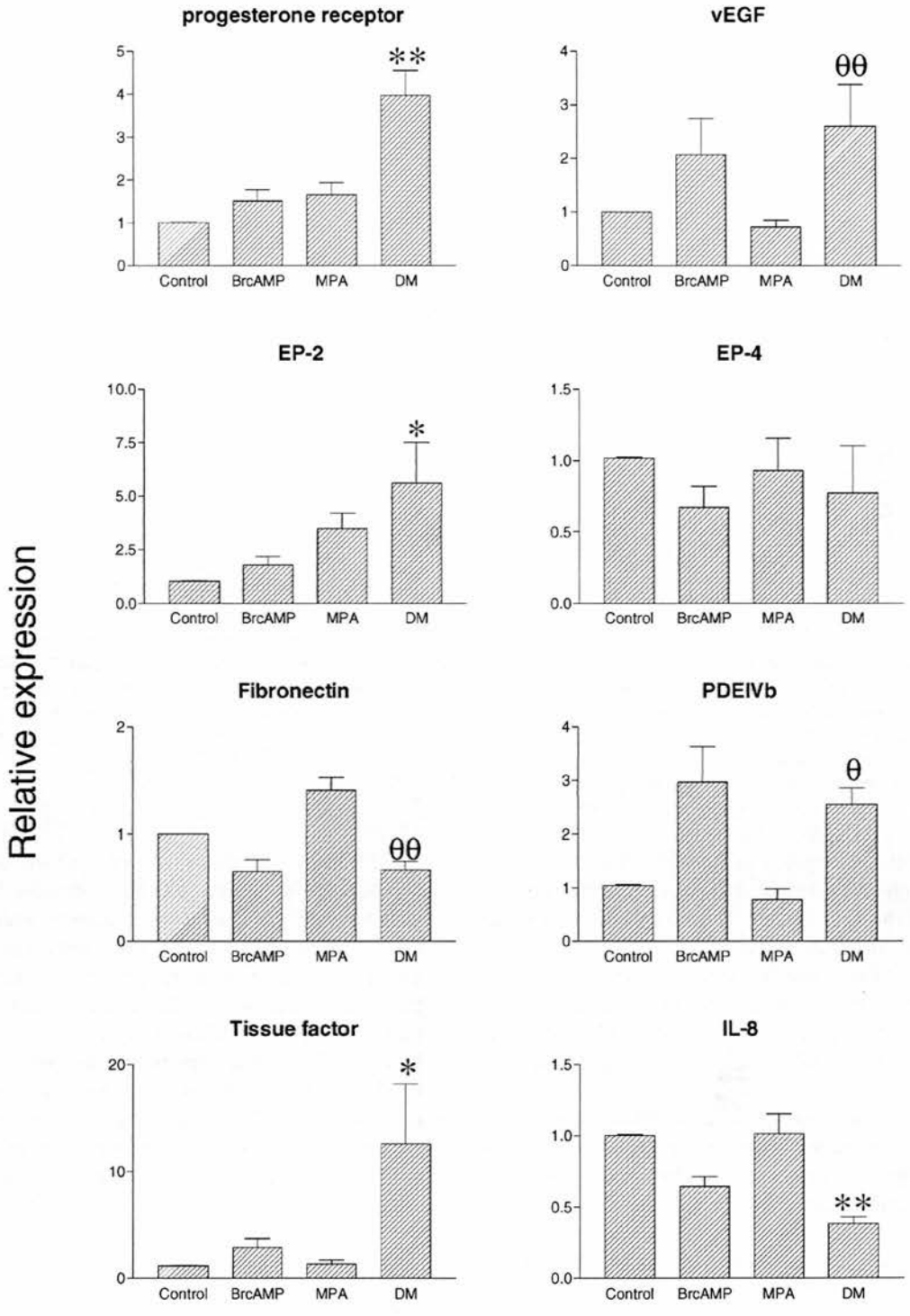

Fig. 4. All cells were originally derived from the cervix of pregnant women ($n = 5$). There is an increase in expression of the EP2 prostaglandin receptor mRNA (but not EP4). Also shown are changes in mRNA for progesterone receptor, vascular endothelial growth factor (vEGF), phosphodiesterase type IVb, fibronectin, tissue factor and IL-8. Bars represent the mean of each treatment group ± S.E.M. Significant differences are denoted as follows: difference from control with $^{**}P < 0.01$ and $^{*}P < 0.05$, and difference from progesterone alone $^{\theta\theta}P < 0.01$, $^{\theta}P < 0.05$.

Prostaglandin dehydrogenase (PGDH) mRNA was found to be increased ($P < 0.05$) in untreated pregnant cervical biopsies relative to non-pregnant cervical biopsies (mean 38.8 ± 21.7 S.E.M. cf. 2.3 ± 1.2 S.E.M., $n = 6$ both groups). However during culture the PGDH mRNA expression diminished and minimal message was found in treated and untreated cultured cervical stromal cells.

4. Discussion

Changes in the consistency of the cervix can be detected in early pregnancy [16] but little is known about the contribution of the various cell types within the cervix to such a change. The fibroblast-like stromal cell of the cervix has been considered an important contributor to physiological changes, both in early pregnancy and at the time of parturition. These cells respond to steroids and initiate collagenolytic pathways [13,17–19] and since they have progesterone receptors, they may play a sentinel role in altering cervical morphology in response to hormone changes. However, if these cells respond to prolonged exposure to progesterone with a change in phenotype, as do cells in the endometrium, then this modified cell would be the target for any signal for the induction of labour.

We show here, for the first time, that fibroblasts from both pregnant and non-pregnant cervix will "decidualise" after treatment with cAMP and progestin as do the fibroblasts derived from endometrium. A significant change in morphology is demonstrated, within a relatively short treatment period, in association with stimulation of IGFBP-1 and PRL mRNA and protein release. Fibroblasts derived from foreskin however do not produce the same response, although mild prolactin (but not IGFBP-1) stimulation is seen as has been reported in skin fibroblasts [20]. Although progesterone appears to be sufficient for the decidual change in vivo, cells in vitro need to be sensitized by elevated intracellular cAMP levels [21]. In vivo, raised intracellular cAMP levels can be brought about by prostaglandin E acting through either EP2 or EP4 receptors to activate the stimulatory G-protein (Gs) or by relaxin inhibiting phosphodiesterase, the enzyme responsible for the catabolism of cAMP [22]. Since one effect of raising cAMP is to stimulate expression of phosphodiesterase (Fig. 4) both of the above pathways will work together in a synergistic fashion to maximise cAMP levels. Other agents involved in decidualisation may also affect phosphodiesterase activity since epidermal growth factor inhibits the activity of an isoform of PDEIV by ERK-2 dependent phosphorylation [23]. Moreover, the increase in phosphodiesterase mRNA (in this instance Type IVb) seen after stimulation with BrcAMP is attenuated by progesterone.

The increase in progesterone receptor mRNA is in accordance with observations in endometrial stromal cells [24].

Tissue factor is known to be stimulated in decidual cells under progesterone regulation, both in vivo and in vitro [25] and is thought to have a role in peri-implantation haemostasis and menstruation. Tissue factor may act as an autocrine enhancer for vEGF stimulation thereby promoting angiogenesis during this time of tissue remodelling [26]. The decidual transformation in endometrium is also associated with an increased production of the angiogenic agent vEGF and its KDR receptor [27] and this process may be responsible for the increased vascularisation of the cervix that is seen during pregnancy. The increase in the EP2 receptor (Fig. 4) may also facilitate vEGF release since PGE stimulates vEGF through the EP2 receptor in synovial fibroblasts [28]. Although there are likely to be other cellular sources of EP receptors in the pregnant cervix, experiments in the baboon show that the EP2 type is predominant [29] although its expression decreases during labour [30].

A significant feature of this decidual change to cervical stromal cells is a decrease in both mRNA and release of IL-8 into culture medium. Since IL-8 is known to be produced by the cervix [31] and attracts neutrophils which are implicated in cervical softening [32], a reduction in IL-8 release by cells during pregnancy is appropriate for the maintenance of a firm, closed cervix up until the time of delivery.

Although decidualisation of the cervical fibroblast has not hitherto been recognised, ectopic decidua has been reported in cervix and ovary during pregnancy [33,34], but there has always been doubt whether this was a manifestation of otherwise unrecognised endometriosis.

Since labour is preceeded by changes within the cervix [35], these findings of the decidual-like changes in cervical stromal cells open a new area for the study of the mechanism of the initiation of labour. The decidualised stromal fibroblast is a potential target for known initiators of ripening, such as PGE and antiprogestins.

Whether the decidualised cervical fibroblast serves to maintain cervical integrity throughout pregnancy, or whether this altered phenotype is involved in the cervical ripening process has yet to be established. A more fundamental point of interest is that the potential for decidual change is likely to be present in all fibroblasts expressing the progesterone receptor. Thus, the phenomenon of ectopic implantation and pregnancy may be reliant on this intrinsic property of fibroblasts anywhere within the reproductive tract, but particularly the fallopian tube. Further assessment of this data should include immunohistochemistry or in situ hybridisation for the presence of decidualisation markers in cervical stromal cells, and ultimately to show decidualised CSC in vivo, although these samples would be extremely difficult to obtain. To elucidate the full nature and purpose of the capacity of cervical fibroblasts to decidualise will require further investigation, but these findings should be considered in future studies on cervical function.

Conflicts of interest

None

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